INVESTIGATING KINESIN-lIKE PROTEIN SMY1P'S FUNCTION IN POLARIZED SECRETION

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ABSTRACT

The budding yeast *Saccharomyces cerevisiae* shows substantial polarity during its growth. Membranes and proteins are constantly transported to the growth sites, almost exclusively mediated by the Myosin motor Myo2p. Myo2p recognizes its cargos by interacting with different cargo-specific adaptors and its function also relies on other accessory proteins. Among them, the kinesin-like protein Smy1p was interesting due to its genetic interactions with *sec2*, *sec4* and *myo2* mutants. However, current models have problems reconciling all the previous findings. Whether Smy1p has a function in polarized secretion and how Smy1p exerts its function are largely unknown. To address these questions, different approaches were employed in this research. First, Smy1p localization was determined and I found it to rely on interactions with Myo2p. Further truncation analysis revealed a complicated localization and expression level regulation. Second, Smy1p protein depletion in *myo2* conditional mutants was found to cause a massive secretion block and this method can be applied to other mutants like *sec2-56*, *sec4-8* as well. Third, I identified over 30 *smy1* mutants in different *myo2* sensitized strains, studying of which will greatly increase our understanding of the mechanism underlying Smy1p's function.

BIOGRAPHICAL SKETCH

Donghao was born in the town of Gushi, located in the middle of China. It is the biggest town in the Henan province, with a population of over 1 million people. In ancient times this area was the heart of the country, a cultural and economical center and the origin of the whole nation. Numerous historical figures were born here and fourteen dynasties built their capitals in Henan. However, nowadays it is only known of great poverty and a large population. Donghao's family was also very poor when he was born, but luckily his parents cared a lot about the child's education and they found his interest in science very early. Therefore they bought all kinds of books and taught him English since 5 years-old, hoping that some day he could go abroad and become a great scientist. Donghao worked hard to fulfill his parents' expectation and got admitted to Peking University, which recruits around 80 students in this province although there are more than 400,000 high school graduates every year. In college, Donghao chose Biology as his major and continued working hard, because he wanted to conduct some really meaningful research in the future. There, he also met a lot of friends and a girl who deeply loved him. Unfortunately, destiny separated them because Donghao decided to go abroad after graduation while she had to stay in China. With a heart filled with pine and thrill, Donghao came to

Cornell and joined in Dr. Anthony Bretscher's lab. The first year of research didn't go very well because he wasn't careful and serious enough, so he got a conditional pass in his A exam. After that, he was in great pressure and tried his best to get some good results. Fortunately, the project in Smy1p started to work really well and he got a master degree by that. Now Donghao has made a new start, trying to gain working experience in the pharmaceutical industry and develop skills in other areas.

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I greatly appreciate the kindly help and patience of my advisor Tony in the past two years. I really enjoyed talking to you about science, and about life. I will keep the lesson in mind.

Also, great thanks to the Bretscher lab members. Thanks to Felipe, who lead me into real science. To Irina and Kirk, who kindly shared their material and ideas. To Ram, for your curiosity and your company in so many nights. To Damien, for the laughter you brought to all of us. To Jess, for your encouragement. To Dave L and Dave H, for the suggestions and help. And to David McDermitt and Janet, for your support to the lab. At last, to Lwin, who helped me a lot in the last few days.

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LIST OF ABBREVIATIONS

AD, *GAL4* activation domain

BD, *GAL4* DNA-binding domain

ccGTD, the region of Myo2p containing the coiled coil and the GTD

ERGIC, ER-Golgi Intermediate Compartment

GAP, GTPase-activating protein(s)

GEF, guanine-nucleotide exchange factor(s)

GTD, the globular tail domain of Myo2p

PI or PIs, phosphoinositides(s)

SR, SRP receptor

SRP, signal recognition particle

TGN, trans-Golgi network

GAL, galactose

CC, coiled-coil

NT, nucleotide

AA, amino acid

RT, room temperature

TS, temperature sensitive

NLS, nuclear localization signal

C.V, column volume

Y2H, yeast two-hybrid

WT, wild type

CHAPTER 1: INTRODUCTION

1.1 Cell Polarity

Polarity is a common feature of most eukaryotic cells. For example, mammalian epithelial cells (Fig 1.1A) have two domains, a basolateral domain and an apical domain. Each domain has different structures and different protein/lipid components. This specific organization allows the cell to carry out its normal function and therefore is essential for cell growth.

To study cell polarity, we use the budding yeast *Saccharomyces cerevisiae* as a model (Fig 1.1B). Budding yeast has a very simple life cycle. At the beginning of each cell cycle, the mother cell chooses a budding site to form a small bud. As the cell cycle proceeds, the bud grows until it reaches certain size, when new cell wall forms between the mother and the daughter cell and separates them, thereby finishing the cell cycle (Sherman, 2002). How budding yeast adopts this highly asymmetric cell shape and achieves such a polarized growth with exceptional accuracy is always intriguing for cell biologists. The basic mechanism underlying this process may very well be conserved and be applied to research in higher organisms.

In the following sections I will briefly describe what we know about the principles of yeast polarity establishment and what problems we are

investigating in our lab, especially in my project.

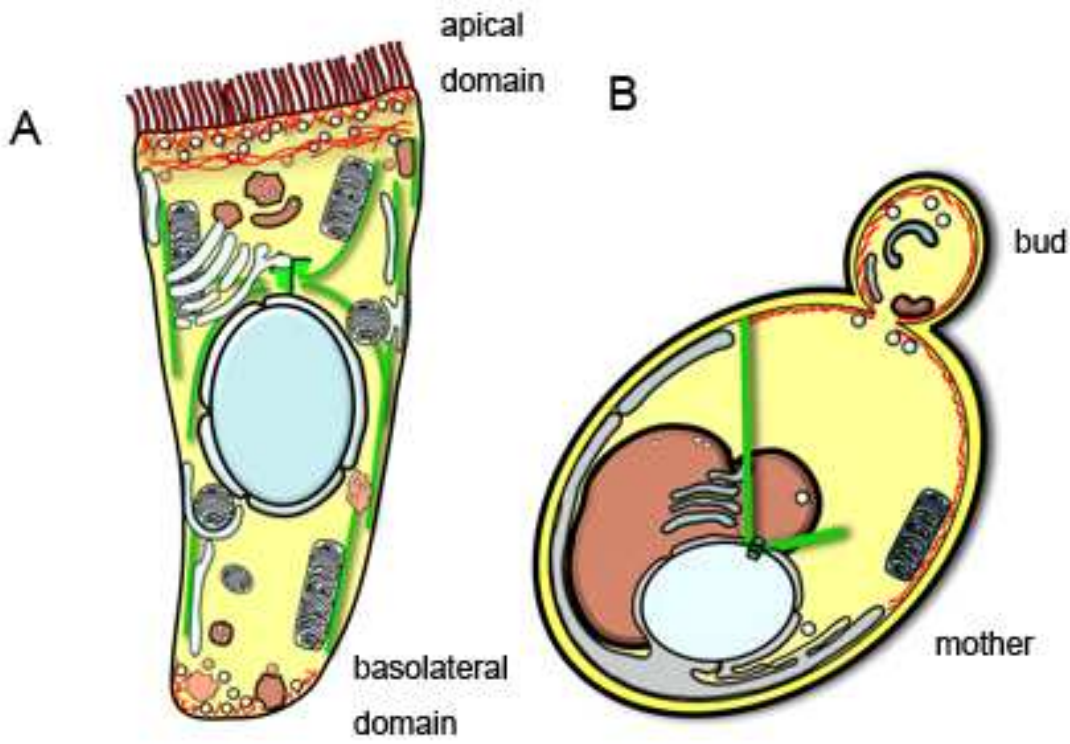


Figure1.1 Polarity is a common feature of eukaryotic cells
(A) mammalian epithelial cells have two domains: apical and basolateral domain
(B) *Saccharomyces cerevisiae* has a bud and a mother cell
Microtubules are shown in green, actin filaments are shown in red.
These schematics are adopted from Felipe Santiago-Tirado's work.

1.2 Polarized Secretion

Polarity establishment requires orderly and timely delivery of protein, lipids and other components. In yeast, multiple membrane compartments, including Golgi, vacuole (Weisman et al., 1990), mitochondria, peroxisome and secretory vesicles, are transported from the mother to the bud during bud emergence, because inheritance of these organelles is very important for the normal function of the newly-formed daughter cell. However, except for mitochondria, most of

these organelles can be generated *de novo*, while the secretion of lipids and protein from ER, Golgi to the plasma membrane (so-called polarized secretion) is essential for bud growth. Therefore, we will focus on that in this thesis.

Continuous delivery of secretory vesicles enables the bud to grow simply in two aspects. First, newly synthesized protein and lipids are added as the secretory membranes fuse with the plasma membrane, providing the substance for the expansion of the surface area. Second, enzymes and other material are secreted outside the cell, remodeling the cell wall and allowing the growth to occur. So how does the cell know what to secrete?

It took almost 40 years to understand the principle dominating this complicated process. First of all, secretory proteins exhibit an N-terminal signal peptide when translated in the cytoplasm, where this peptide is recognized by SRP complexes. SRP complexes then bind to this peptide and stall the translation, until captured by the SRP receptor located on the ER membrane. There, as the translocon is assembled, translation of secretory protein is resumed and the resulting peptide is translated and then folded and modified in ER lumen (membrane proteins work slightly differently). Second, these newly synthesized proteins are enclosed in membrane and coated by the COPII complex, which then buds from the ER and is transported to the Golgi complex. Note that in budding yeast,

since cortical and perinuclear ER are scattered, there is no obvious ER exit site like higher organisms. Also, the ER-Golgi intermediate complex (Hauri and Schweizer, 1992) cannot be clearly discriminated from early cisterna because the Golgi complex is scattered as well. As a result, transport between the ER and the Golgi actually happens through diffusion in yeast, while in mammalian cells active transport is involved. After secretory proteins reach the Golgi complex, they are subject to additional modifications as the cisternae matures into late Golgi and the Trans-Golgi Network (TGN), where sorting occurs (Losev et al., 2006). However, it is still unclear how vesicles are packaged at the TGN, except for the exomer (Wang et al., 2006) that specifically coats certain vesicles carrying cargos like Chs3p (a chitin synthase). At last, TGN gradually mature and break into secretory vesicles, containing all the protein and lipids needed for the new bud, and are transported to the growth site. There, with the help of the tethering complex exocyst (Guo et al., 1999), which consists of 8 different proteins (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p), these vesicles are docked at the plasma membrane, and subsequently fuse, driven by SNARE proteins.

As you can see, polarized secretion involves active transport of cargoes into the bud. Then how could this directional transport be possible?

1.3 Actin Cytoskeleton

The key to polarized transport is to organize a polarized cytoskeleton. In

budding yeast, the actin cytoskeleton is the dominant transportation track (Adams and Pringle, 1984), in contrast to higher organisms where microtubules and actin are both important. The actin cytoskeleton in yeast is further divided into three forms: actin patches, actin cables and the cytokinetic ring (Kilmartin and Adams, 1984). Actin patches are involved in endocytosis (Kaksonen et al., 2003) and therefore is beyond the scope of this study. I will talk more about actin cables below.

Actin monomers can easily self-polymerize into filamentous structure *in vitro*. However, *in vivo* actin monomers are bound to profilin, which prevents self-assembly and directs assembly by regulated processes (Haarer et al., 1990). The regulators that cooperate with profilin to form actin cables are formin proteins (Imamura et al., 1997), actin nucleators that contain catalytic FH1 and FH2 domains (Pruyne et al., 2002). In yeast there are two of them, Bni1p and Bnr1p, which are partially redundant. Bni1p localizes to the bud tip, while Bnr1p localizes to the bud neck. Once recruited to the right place, they are activated and start nucleating actin assembly by interacting with profilin-bound actin monomers. Formin activity is tightly regulated, since mislocalized formin activity has adverse effect on cell growth (Liu et al., 2012). At the same time, the actin filaments formed are always of roughly the same length (0.3-0.5μm), but bundled by fimbrin (Adams et al., 1991) and stabilized by tropomyosin (Liu and Bretscher, 1989) to form the long actin cables.

As formins stay at the barbed end of the actin filaments and are anchored on the plasma membrane, the cell is able to have a polarized actin cytoskeleton (Fig 1.3), which provides the track for active transport.

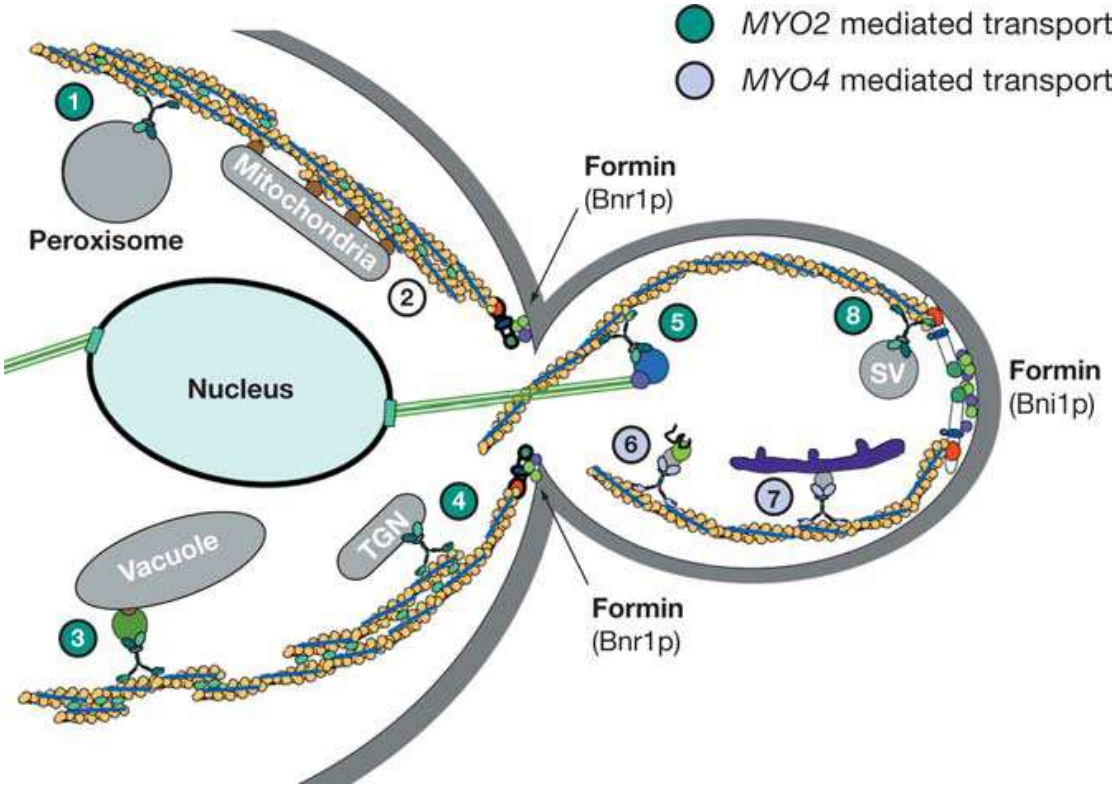


Figure 1.3 Actin cable Assembly in budding yeast. This is adopted from a review (Pruyne et al., 2004). Formins are actin nucleators. Bni1p localizes to the bud tip while Bnr1p localizes to the bud neck. Actin filaments nucleated from them are decorated by fimbrin and tropomyosin to form the long actin cables.

1.4 MyosinV Myo2p

Polarized transport is not possible without the motor proteins that travel along actin cables. In budding yeast, the myosin-V family motor Myo2p is the main motor that carries out most of the transport, including secretory membranes, different organelles and astral microtubule (Yin et al., 2000). Therefore, a lot of our current understanding of yeast polarity comes from study of this protein.

As a myosin-V family motor, Myo2p can be divided into four different domains: the head domain, the neck domain containing IQ motifs, a coiled-coil region and the globular tail domain (GTD) (Fig 1.4A).

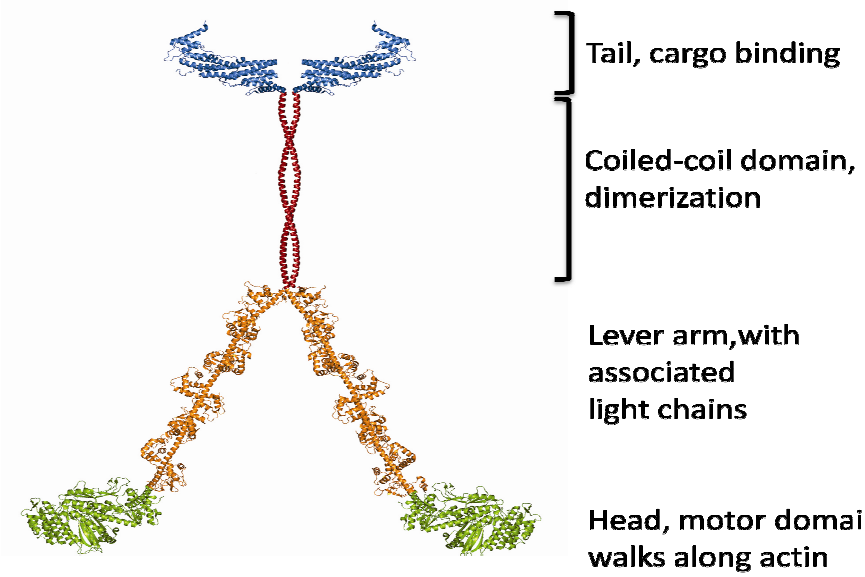


Figure 1.4 (A) Myo2p is a Myosin V family motor This diagram was made by graduate student Kirk Donovan. It shows different domains of Myo2p.

The head domain, also called the motor domain, is conserved through the myosin-V family, which binds actin cables and has ATPase activity. Myosin-Vs have a high duty-cycle, which means over 90% of the time the motor domain remains bound to actin (De La Cruz et al., 1999). Therefore, most of them are processive motors, walking along actin cable for several steps before falling off. However, Myo2p was considered an unusual myosin-V because in previous *in vitro* experiments it was non-processive (Hodges et al., 2009). Despite that, Myo2p is a processive motor *in vivo*, as the motor itself could reach the bud tip when detached from its secretory cargo in *myo2* conditional mutants with mutations on their tail domain (Schott et al., 1999).

The neck domain with IQ motifs is responsible for light chain and calmodulin binding (Espindola et al., 1992), and recently found to be also the binding site of Sro7p (Rossi and Brennwald, 2011). Myo2p has 6 IQ motifs, with different preference to different binding partners. However, this domain is not essential, as a 0-IQ mutant generated in our lab is still partially functional (Schott et al., 2002). From the same work, we also found that the number of IQ motifs determines the transport rate of the motor, which normally involves steps of 36 nm for 6 IQ motifs. Therefore, the IQ motifs are called the lever arm.

The coiled-coil region mainly dimerizes this molecule, but is also implicated in binding to Rab proteins (Roland et al., 2011; Santiago-Tirado et al., 2011). Mammalian Myosin-V has three coiled-coil regions, while Myo2p only has one. That's why some conserved residues in mammalian myosin-Vs cannot be found in yeast.

The globular tail domain (GTD) of Myo2p is vital for its function, since it is the cargo-binding domain (Schott et al., 1999). With its structure solved (Pashkova et al., 2006), and different point mutations available, the field has a fairly good understanding of its organization and function (Fig 1.4B). The GTD can be divided into sub-domain 1 and sub-domain 2. Sub-domain 1 is mainly responsible for binding to vacuole and mitochondria, while sub-domain 2 mainly binds secretory compartments (Pashkova et al., 2005). Some of the important surface residues that

affect Rab binding have been revealed (Eves et al., 2012), which will be covered in the following chapters.

How could Myo2p recognize so many different cargos with only one tail domain? The answer lies in adaptor proteins.

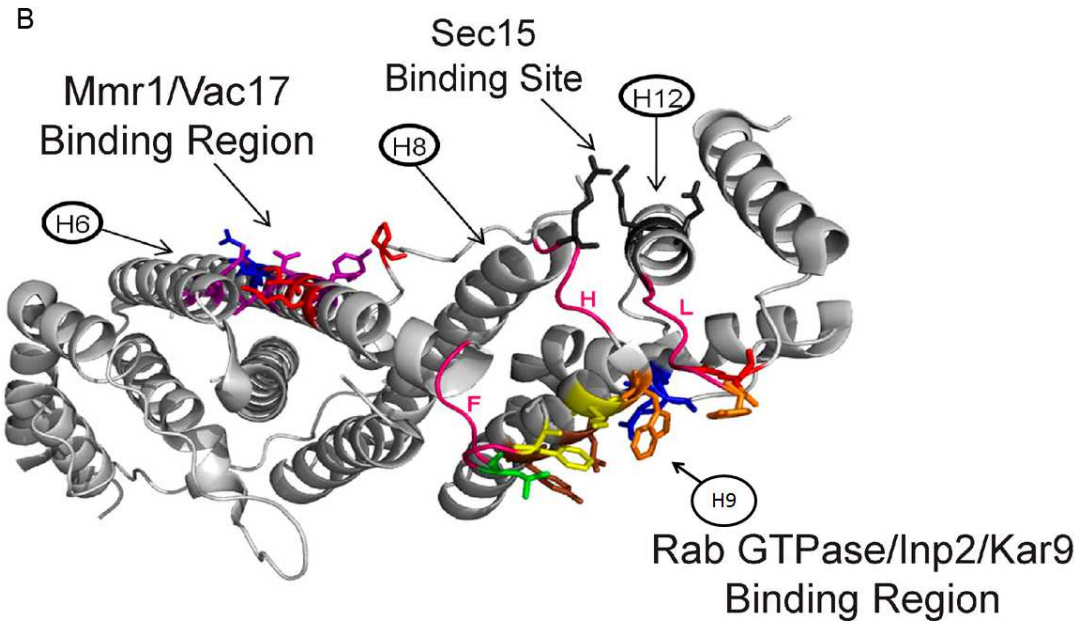


Figure 1.4 (B) Myo2p tail structure and adaptor binding site. This is adopted from a most recent paper (Eves et al., 2012). Different binding sites for different adaptors are shown. The Rab binding site is mainly around helix 9, which overlaps with Inp2 and Kar9 binding site.

1.5 Adaptors

The idea that the myosin motor binds its cargo through an intermediate adaptor protein has been proposed for many years. For example, vacuole adaptors are Vac8p-Vac17p (Ishikawa et al., 2003) and mitochondria uses Mmr1p and Ypt11p (Itoh et al., 2004). In mammalian cells, melanosomes need Rab27a to be transported by myosin Va (Strom et al., 2002). Rab proteins, a family of small GTPase that can switch between GDP and GTP bound state, are perfect candidates for regulating complicated

membrane trafficking, by binding to motors proteins. Indeed, in yeast, it was found that different organelles employ different adaptors, of which Rab proteins are commonly used. We are more interested in the post-Golgi secretion process, where a Rab cascade (Mizuno-Yamasaki et al., 2010) is involved (Fig 1.5).

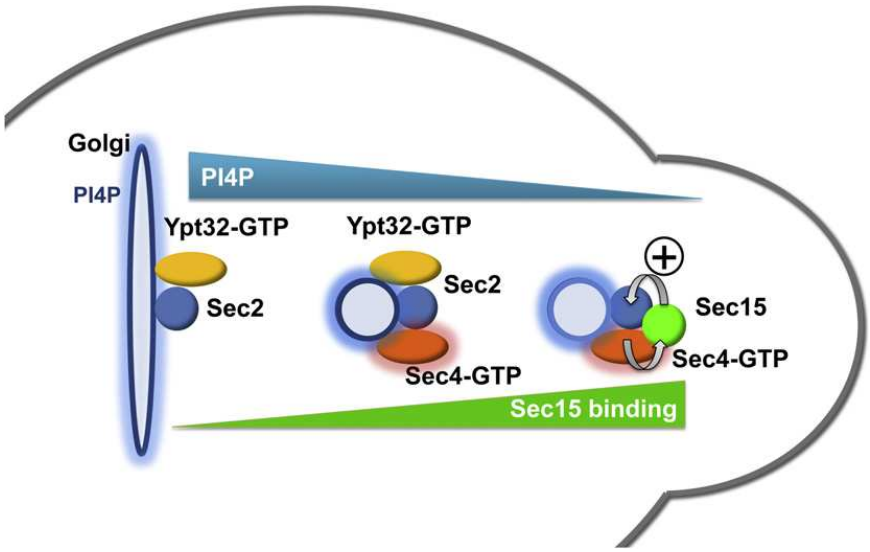


Figure 1.5 Rab cascade happens in late secretion. This is adopted from Novick group's paper (Mizuno-Yamasaki et al., 2010). Sec2p is in blue, Ypt32p is in yellow, Sec4p is in red and Sec15p is in green. As PI4P level drops, Sec15p gradually displaces Ypt32p.

Rab proteins Ypt31p/Ypt32p are localized on late Golgi cisternae and the TGN, where they serve as the Myo2p receptor. At the same time, the Novick lab found that, by binding to PI4P and Ypt31p/Ypt32p, Rab GEF Sec2p also localizes to these compartments. Sec2p may also contribute to Myo2p binding, although the significance of this interaction has not been confirmed (unpublished work from Felipe Santiago-Tirado). As

these membrane compartments are moved towards the bud, they gradually mature and the Rab GTPase Sec4p is recruited and activated by Sec2p. Sec4p has been long proposed to be the Myo2p receptor on secretory vesicle. However, it was not confirmed until Felipe Santiago-Tirado in our lab used a series of *myo2* tail mutants that lack the interaction with Sec4p and characterized their phenotype (Santiago-Tirado et al., 2011). What's more, Felipe also found that increasing PI4P level on secretory compartments could partially suppress some *myo2* tail mutants, suggesting a coincidence detection model where PI4P serves as part of the receptor. The only mystery left is how Myo2p binds to PI4P when no direct interaction could be found. One hypothesis favored by our lab is that an intermediate factor physically links Myo2p and PI4P, though such a protein has not yet been identified. Once activated, Sec4p binds to its effecter Sec15p (Ortiz et al., 2002), which in turn displaces Ypt31p/Ypt32p (Mizuno-Yamasaki et al., 2010). Sec15p is part of the exocyst complex and it also binds to Myo2p. This interaction was recently found to affect Myo2p binding and secretion, although very weakly (Jin et al., 2011).

With all these findings, it seems that how Myo2p transports secretory compartments is quite clear. However, there are still some mysteries to be solved, of which Smy1p is one.

1.6 Smy1p

Kinesin-like protein Smy1p was identified in 1992 by the Lillie and Brown group in a multi-copy suppressor screen for the Myo2p head mutant *myo2-66*, which is the first *myo2* mutant (Lillie and Brown, 1992). Later it was discovered to also suppress most conditional *myo2* tail mutants generated by a former graduate student Daniel Schott in our lab. *smy1Δ* cells show a very modest phenotype, but *smy1Δ* is synthetic lethal with *sec2-56*, *sec4-8* and *myo2* tail conditional mutants (except for *myo2-14*), indicating its involvement in polarized secretion. The authors tried to correlate Smy1p function to its kinesin activity, proposing that Smy1p travels down the microtubule to polarized growth site and brings Myo2p to the right destination. Indeed, Smy1p interacts with Myo2GTD through its tail domain (Beningo et al., 2000). Smy1p overexpression causes Myo2p to be hyperpolarized in WT cells and restores Myo2p polarization in the *myo2* mutants (Beningo et al., 2000). However, using the microtubule destabilizing drug nocodazole, the same group found that Smy1p function doesn't need microtubules but relies on functional actin cytoskeleton instead (Lillie and Brown, 1998). Later it became clear that Smy1p is a passenger on secretory vesicles moved by Myo2p (Chesarone-Cataldo et al., 2011). How does Smy1p exert its function? Two recent models were proposed.

In the first one, the authors used quantum dots to mimic secretory vesicles and *in vitro* assays to show that Myo2p cannot undertake

continuously long-range movement along actin bundles without Smy1p. In addition, they showed that Smy1p can randomly diffuse along actin bundles, indicating a weak interaction between them. Lastly, they claimed that Smy1p co-localizes with Sec4p, a vesicle marker. As a result, Smy1p was proposed to bind to secretory vesicles as well as Myo2p, and functions as an electrostatic tether to actin cable that enhances the processivity of Myo2p (Hodges et al., 2009) (Fig 1.6A). However, this model is questionable because we know Myo2p should be a processive motor *in vivo* (Schott et al., 1999) and a recent paper from the same group proposed that adding tropomyosin to the system would make Myo2p processive (Hodges et al., 2012). Besides, it is inconsistent with our finding that Smy1p overexpression also suppresses *myo2* tail conditional mutants, which are detached from secretory vesicles at the restrictive temperature.

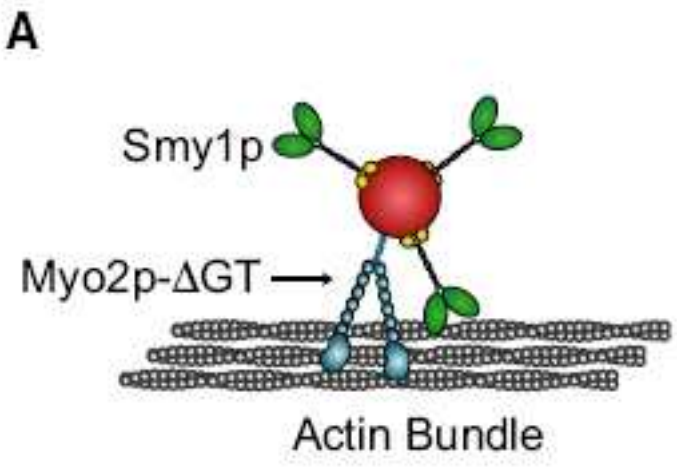


Figure 1.6 (A) Smy1p works as electro static tether and helps Myo2p walk on actin bundle. This is adopted from Trybus group's paper (Hodges et al., 2009). The green motor is Smy1p, red ball is Qdot, and blue motor is Myo2p.

The second group suggested Smy1p is a formin regulator that specifically suppresses the activity of Bnr1p, so that disrupting Smy1p function causes abnormal actin cytoskeleton organization (Chesarone-Cataldo et al., 2011) (Fig 1.6B). The authors first used an *in vitro* actin assembly assay to demonstrate that when a Smy1p fragment is added, the actin nucleating activity of Bnr1p is largely suppressed. Then, they showed that *smy1Δ* cells have defects in actin structure. That is, actin cables overgrow and kink, which is not seen when *BNR1* is deleted. To further suggest an interaction between Smy1p and Bnr1p, imaging experiments revealed that Smy1p marked vesicles frequently pause at the bud neck. However, this hypothesis is weak in three aspects. First, they only showed that truncated but not full-length Smy1p has a strong effect on the actin assembly activity of Bnr1p. Second, they failed to discriminate Smy1p marked vesicles from other vesicles, in order to rule out the possibility that pausing at the bud neck is a common behavior of all vesicles due to the limited space. Lastly and most importantly, this model is difficult to reconcile with the previous data, i.e. how Smy1p overexpression could suppress *myo2* mutants.

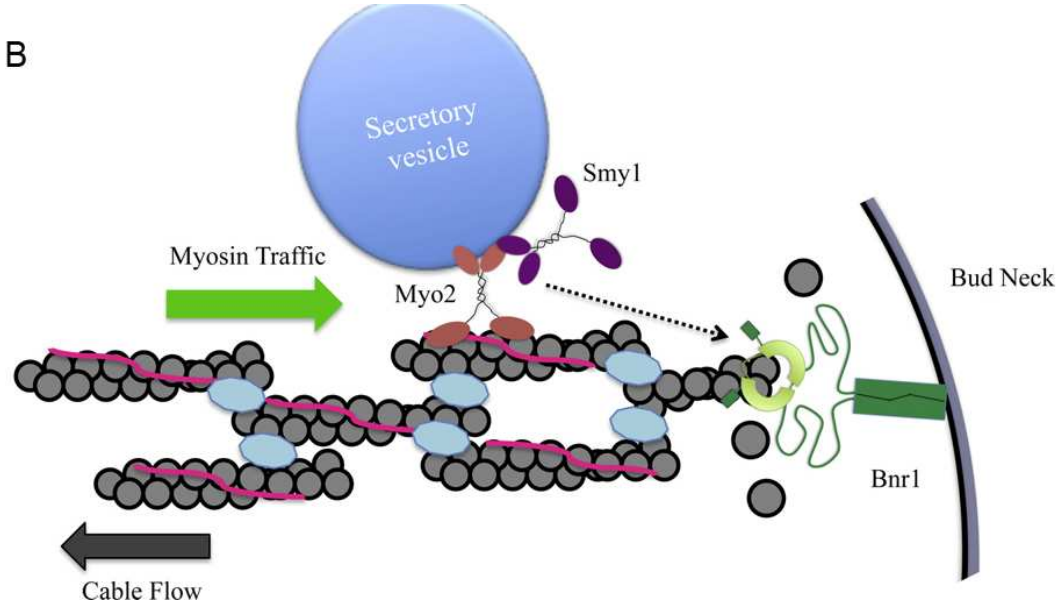


Figure 1.6 (B) Smy1 was proposed to be a Bnr1p damper. Adopted form Melissa Chesarone-Cataldo et al. 2011, this figure shows that Smy1p is a vesicle passenger and travels to the bud neck, where it functions as a formin regulator.

In summary, despite all the genetic interactions and phenotypes identified in the last two decades, Smy1p function remains a mystery. So I set out to explore this mystery when I joined the Bretscher lab, and in the following chapters I will present the data I collected, which hopefully could deepen our understanding of this protein.

CHAPTER 2: STUDY OF SMY1P LOCALIZATION

Introduction

Smy1p is interesting because it is a kinesin-like protein. Like most kinesins, it has three different domains: a head/motor domain, a coiled-coil region and a tail domain, presumably functioning as a dimer. Kinesins are microtubule-based anterograde motors, that is, they move towards the plus end of a microtubule in the cell (traditionally it is believed so, but people have found kinesins moving in the opposite direction). As expected, Smy1p was shown by immunofluorescence to be polarized at the bud tip and bud neck (Lillie and Brown, 1994). Surprisingly, this localization is dependent on the actin cytoskeleton but not microtubules. Therefore, how a kinesin-like protein could travel along actin cables was an interesting question for the early investigators. An important hint comes from the observation that Smy1p overexpression restores Myo2p localization in *myo2-66* cells at the restrictive temperature, and hyperpolarizes Myo2p in WT cells. So it is tempting to think that there is some sort of interaction between them. Indeed, an interaction between Smy1p and Myo2p GTD was found through yeast two-hybrid (Beningo et al., 2000). And the Myo2p interacting domain of Smy1p was mapped to the C-terminal region or the tail domain. As a result, one would naturally assume that Smy1p binds to Myo2p and moves to the polarized growth sites together,

although there is no direct evidence to support this idea.

This was unquestioned until the Trybus group proposed Smy1p is actually attached to secretory vesicles and acts as an electro-static tether to actin cables. Their evidence is as follows: First, no physical interaction between Myo2p and Smy1p can be detected by immuno-precipitation, even using purified proteins. Second, Smy1p not only travels together with Myo2p, but also co-localizes with Sec4p, a vesicle marker. So it would be reasonable to suggest that Smy1p may be just a passenger protein on secretory vesicles.

This paper raised my interest to ask how exactly Smy1p achieves its localization. To answer this question, I used fluorescence microscopy to study GFP-tagged Smy1p localization in different mutants.

Material and Method

Vectors and Cloning

Standard molecular cloning techniques were employed, which can be found in “Molecular Cloning: A Laboratory Manual, Third Edition, CSHL Press, 2001”. Restrictive enzymes and DNA polymerase were used according to the instructions in either NEB or Roche’s user manual.

Plasmids were all from the lab database or slightly modified. The vectors used include pRS415, pHL-3GFP, pRS416-3mCherry, pRS306 myo2-3GFP, pGADT7 myo2-cctail, pAS2-smy1, pBridge-sec4ΔC, pBridge-sec4ΔC+NLS-smy1 and Longtine plasmid (3HA).

Yeast strains and transformation

Yeast strains used are WT (BY), *myo2-12*(BY), *myo2-14*(BY), *myo2-16*(BY), *myo2-24*(BY), *sec2-56*(Novick lab), *sec4-8*(Novick lab) and Y strains for Y2H (Haiyuan Yu lab). Yeast transformation was done either using the ez-Frozen-EZ Yeast Transformation Kit (ZYMO Research) or following the LiAc protocol as described (Gietz and Woods, 2002).

Fluorescence microscopy

Strains were grown in 5ml of standard YPD or SD medium until OD_{600} =0.8 to 1.2, spun down, washed once with same volume of ddH_2O and resuspended in 0.5ml SD-Trp medium. After that, an agarose pad was made, by spotting ~200ul pre-warmed 1% agarose containing SD-Trp medium onto each well of a 10-well glass slip, and covered by a coverslip. 5 mins later, the coverslip was removed, and one can spot 3.5ul of sample onto each well. Then, another coverslip was put atop to seal the agarose pad, and the cells would be ready for imaging.

To visualize the cells, a fluorescence confocal microscope (3i system) was used, following the standard operating manual.

Yeast two-hybrid

In this experiment, Y8830 strain was transformed with the AD-myo2 cctail plasmid, while the Y8910 strain was transformed with BD-sec4 or BD-smy1 plasmid. After that, strains were mated on YPD plates for 5h at 30℃ to combine the two parts. The resulting diploids were grown to

mid-log phase and normalized to OD_{600}=0.5. A serial dilution assay was performed (5X dilution), and spotted onto SD–Leu-Trp (2DO), SD-Leu-Trp-His (3DO) and SD-Leu-Trp-His-Ade (4DO) plates, incubated at either 26℃ or 35℃.

Fractionation assay

Basically, cells were grown in 5ml of YPD medium at 26℃ overnight, and expanded to 25ml the next day to mid-log phase. OD_{600} was measured and 25 OD unit (OD*ml) of cells were taken, spun down, washed once with 25ml chilled killing buffer (50mM Tris pH7.5, 1mM EDTA, 1mM $MgCl_2$, 100mM sorbitol, 10mM NaN_3). A second wash was performed with 5ml spheroplasting buffer (20mM Tris pH7.5, 1.2M sorbitol, 25mM βME). The cells were resuspended in 1ml digesting buffer (spherolasting buffer + 125ul 1mg/ml zymolyase), incubate 30 min at 37℃. After that, the cells were spun down at 1,000xg for 5min using a clinical centrifuge and the supernatant was discarded. The pellet was resuspended in 1ml lysis buffer (50mM Tris pH7.5, 1mM EDTA, 1mM $MgCl_2$, 100mM sorbitol) with protease inhibitor and transferred to a 5ml homogenizer. Tubes were washed twice with 1ml lysis buffer, so there would be 3ml in total. The cells were homogenized 10~15 times on ice, with 120ul taken as “Input”. The rest was put in 3 equal aliquots and spun down at 600xg for 5min in a clinical centrifuge at 4℃. The supernatant was the “S6” fraction and the pellet was the “P6” fraction. Two tubes of the “S6”

fraction were spun again at 13,000 for 5min. The supernatant was the “S13” fraction and the pellet was the “P13” fraction. One “S13” fraction was put into ultracentrifuge tube, spun at 100,000xg for 1h at 4℃. Here we got the “P100” and the “S100” fractions. As a control, I divided “S100” into two aliquots, and added 1% triton X-100 to one of them, which would elute off membrane bound proteins. After that, the same volume of lysis buffer as the “S” fraction was added into the “P” fraction to resuspend the pellet. At last, 200ul of sample and 50ul 5x SDS loading buffer were mixed, vortexed and boiled for 5min. I ran a SDS-PAGE for each fraction, followed by western blots using anti-HA, anti-Sec4p and anti-Myo2 tail antibody.

Results

2.1 Smy1p is a passenger protein on secretory vesicle

Smy1p is known to polarize to the bud tip and bud neck, but we need an accurate way to visualize how it is transported to these growth sites. Therefore, live imaging using a fluorescence microscope was employed. WT cells were transformed with pHL Smy1-3GFP plasmid, which expresses Smy1p tagged with 3GFP under its own promotor. When I looked at these cells at room temperature under microscope, I found a bright cap at the bud tip in those small and medium budded cells. What’s more important, bright dots are constantly generated in the mother cell

and rapidly transported into the bud (Fig 2.1A). Later, when I double labeled Smy1p-3mCherry and GFP-Sec4, I found they co-localize (Fig2.1B). Taken together, these data suggest Smy1p is a passenger protein on secretory vesicles.

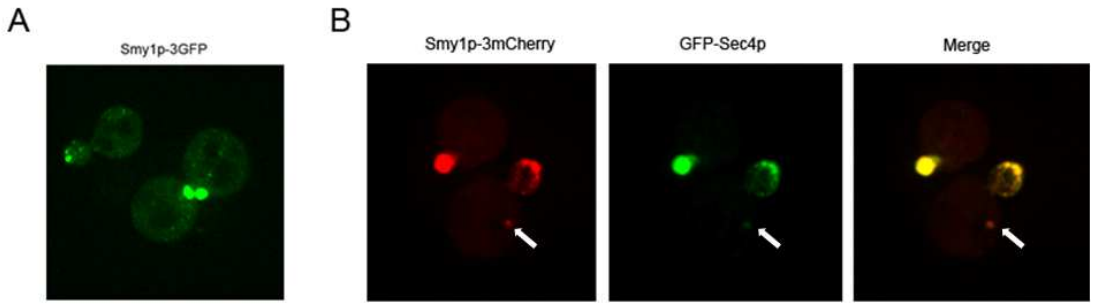


Fig 2.1 Smy1p is a passenger protein on secretory vesicle.
(A) Smy1p-3GFP in WT cells at RT.
(B) Smy1p-3mCherry and GFP-Sec4p co-localize in WT cells at RT (white arrow points to a vesicle).

2.2 Smy1p loses its localization in *myo2*, *sec2* and *sec4* mutants

To answer the question what factors affect Smy1p localization, I decided to take advantage of the mutants that will separate Myo2p from secretory vesicles. First, I used *myo2* tail conditional mutants, including *myo2-12*, *myo2-14*, *myo2-16* and *myo2-24*. At room temperature, Sec4p staining in both *myo2-12* and *myo2-16* is largely depolarized (this research), while at restrictive temperature, the staining is totally diffuse (Schott et al., 1999). *myo2-14* is rather special because it has a small truncation at the C-terminal which disrupts its interaction with Smy1p, but the Sec4p staining remains normal at room temperature. *myo-24* has the same C-terminal truncation as *myo2-14*, but no other mutations

(From Dr. Irina Chernyakov), so that other factors can be excluded. In these mutants, I expressed Smy1p-3GFP and Sec2p-RFP as vesicle marker (Smy1p-3mCherry and GFP-Sec4p for *myo2-12*) to track their localization. As expected, at room temperature, in *myo2-14* and *myo2-24*, Smy1p staining is diffuse while Sec4p remains polarized (Fig 2.2A), suggesting Myo2p binding is necessary for the correct localization of Smy1p. Surprisingly, although Myo2p is polarized, Smy1p is largely depolarized in *myo2-12* and *myo2-16* cells (Fig 2.2A, B and C). To investigate this result, later I performed a yeast two-hybrid experiment and found *myo2-16* has a weakened interaction with Smy1p (Fig 2.2D). Therefore, Smy1p localization is highly sensitive to its interaction with Myo2p. However, for *myo2-12*, I still have no convincing answer. I would suggest that Smy1p interacts with Myo2p only on secretory vesicles, and Myo2p binding alone is not sufficient for Smy1p localization.

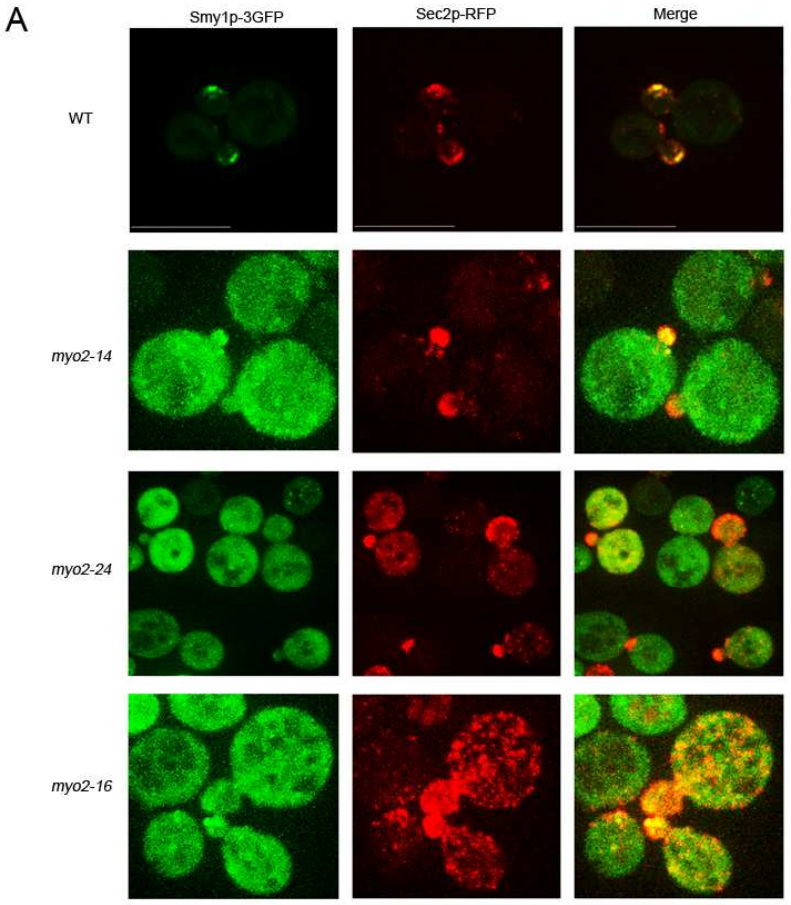
In addition to that, I also studied the effect of *sec* mutants on Smy1p localization. Before, it was believed that Myo2p would always polarize by itself when it is detached from secretory vesicles. So in *sec2-56* and *sec4-8*, which accumulate vesicles all over the cell at the restrictive temperature, I would be able to tell if Smy1p binds to Myo2p or the vesicles. The result was quite unexpected yet reasonable. At room temperature, Smy1p-3mCherry and Myo2p-3GFP are both well polarized. In contrast, in *sec2-56* and *sec4-8* cells, when shifted to 35℃ for 20min,

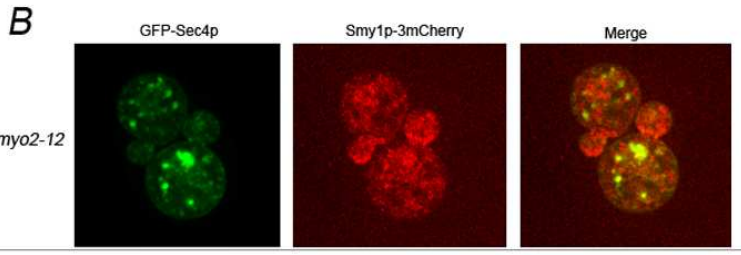
both Myo2p and Smy1p staining became diffuse (Fig 2.2E). This was the first time that we observed a depolarized Myo2p when actin structure is still intact (data not shown), which means that Myo2p probably needs an initiation step so that it can continue walking along actin cables, which is tightly related to the function of Sec4p. For Smy1p, again this is a strong evidence that Smy1p doesn't bind to secretory vesicle but needs Myo2p to correctly localize.

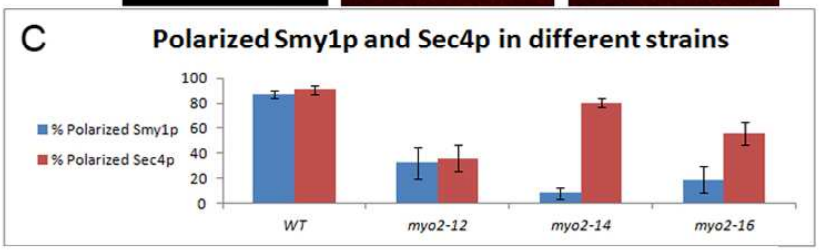
2.3 Smy1p truncations localize in various ways

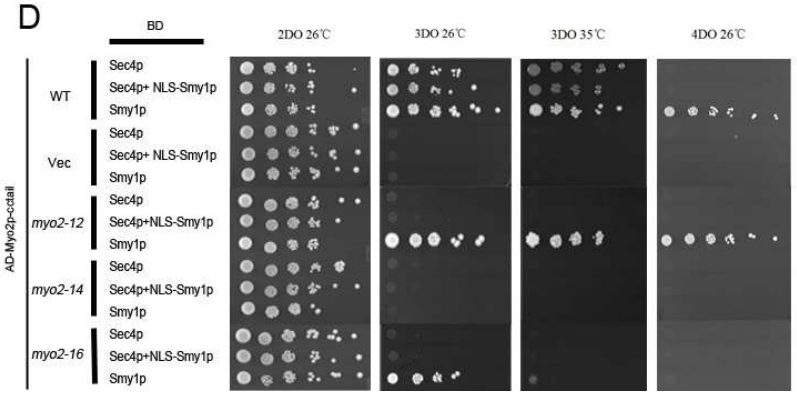
Smy1p has three domains: a head domain, a coiled-coil region and a tail domain. Previous data suggests the tail domain to be the Myo2 binding domain (MBD), and Smy1p truncation without this region lost its localization as well as function (Beningo et al., 2000). So it is intriguing to ask how each domain affects Smy1p localization. An easy strategy is to construct truncations with different combinations of domains and to study their localization respectively (Fig 2.3A).

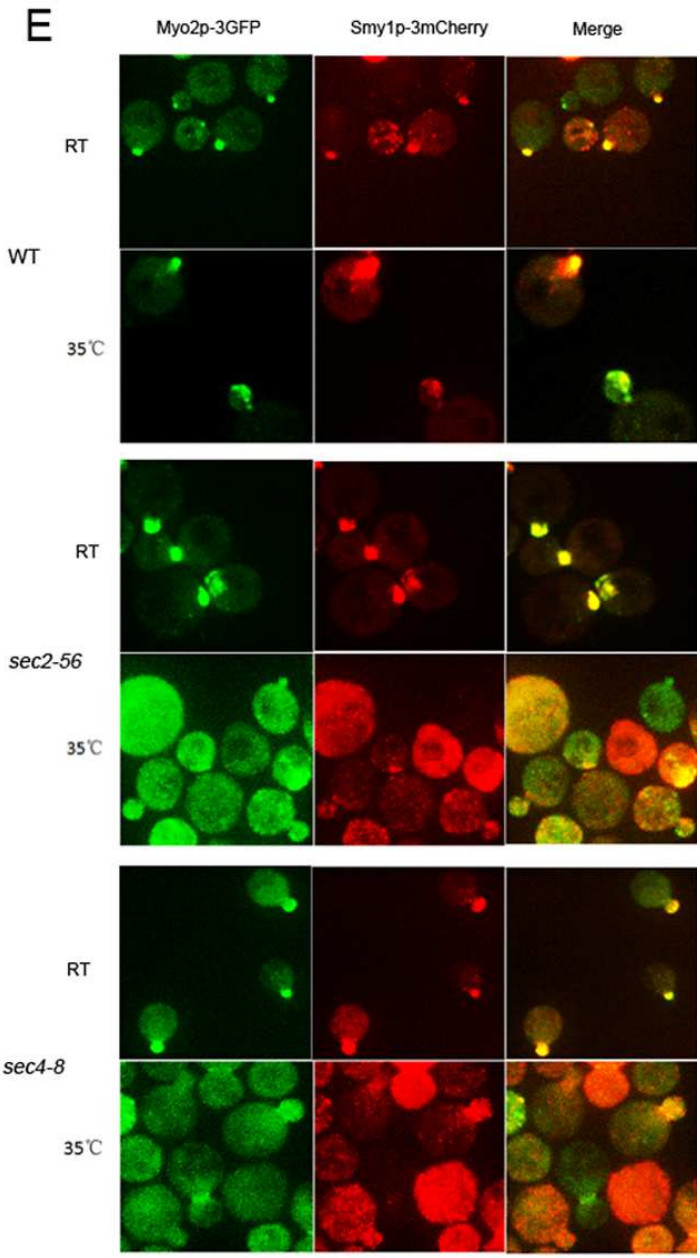
In total, I made 7 different truncations, under the control of the native promotor, tagged with 3GFP. The first thing I tested was if these constructs are functional or toxic. In *myo2-16 smy1Δ* + pRS316 *SMY1* cells, replacing the WT copy with any truncation construct by plasmid shuffling didn't rescue the synthetic lethality. That means none of them


A
Smy1p-3GFP
Sec2p-RFP
Merge
WT
myo2-14
myo2-24
myo2-16


B
GFP-Sec4p
Smy1p-3mCherry
Merge
myo2-12


C
Polarized Smy1p and Sec4p in different strains
% Polarized Smy1p
% Polarized Sec4p
100
80
60
40
20
0
WT
myo2-12
myo2-14
myo2-16


D
BD
2DO 26℃
3DO 26℃
3DO 35℃
4DO 26℃
AD-Myo2p-c-tail
WT
Vec
myo2-12
myo2-14
myo2-16
Sec4p
Sec4p+ NLS-Smy1p
Smy1p


E
Myo2p-3GFP
Smy1p-3mCherry
Merge
RT
WT
35℃
RT
sec2-56
35℃
RT
sec4-8
35℃

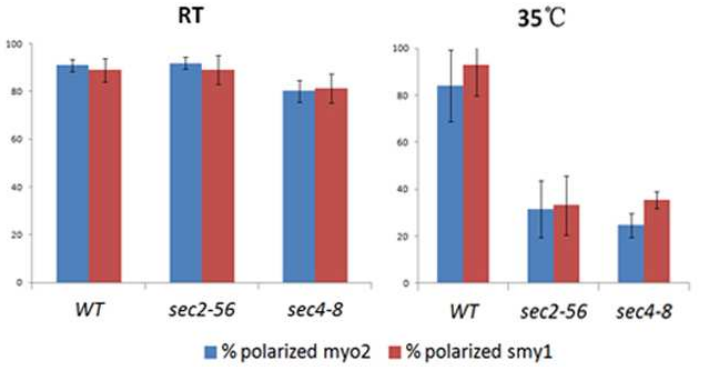

RT
35℃
100
80
60
40
20
0
WT
sec2-56
sec4-8
% polarized myo2
% polarized smy1

Fig 2.2 Smy1p localization in different mutants.

(A) Smy1p-3GFP and Sec2p-RFP in WT, *myo2-14*, *myo2-16* and myo2-24 cells at RT. Smy1-3GFP was expressed under its own promotor on a CEN plasmid. Smy1p localization is lost in *myo2-14* and *myo2-24*, while Sec2p remains polarized. In *myo2-16* both of them are depolarized.

(B) Smy1p-3mCherry and GFP-Sec4p in *myo2-12* at RT. GFP-Sec4p is expressed under the Yop1 promotor on a CEN plasmid. Smy1p-3mCherry is the same as the GFP version in A. Sec4p forms bright spots in the mother cell, indicating a secretion block. Smy1p staining is diffuse.

(C) Smy1p and Sec4p polarization is quantified in *myo2* tail mutants. Data were collected based on imaging of Smy1p-3mCherry and GFP-Sec4p. N=3.

(D) Yeast two-hybrid to test the interaction between different *myo2* cctail and Smy1p or Sec4p. WT cctail interact with Sec4p weakly and Smy1p strongly at all temperature. All tail mutants have lost their interaction with Sec4p, even when NLS-Smy1p is expressed (not verified due to lack of Smy1p antibody). *myo2-14* totally loses its interaction with Smy1p, while *myo2-16* is partially affected (weak on 3DO at high temperature, no growth on 4DO).

(E) Live imaging of Myo2p-3GFP and Smy1p-3mCherry in *sec2-56* and *sec4-8* cells at RT and 35℃. At restrictive temperature, both localizations are lost. Quantification is based on three independent experiment (N=3). Vesicle marker was not available here as Sec2p and Sec4p cannot be used. GFP-Snc1p and RFP-Snc2p were both too dim to give any clear vesicle staining.

is fully functional as the WT allele. Also, expressing them doesn't have any obvious effect on the cell growth in WT and *myo2-16* cells (data not shown). With that clear, I expressed them in WT cells at room temperature, and found all of them showed different localization patterns (Fig 2.3B). Smy1p without its tail doesn't localize to the bud tip as expected, but instead goes to the vacuole and presumably is degraded, as the staining is very dim. In comparison, Smy1p without its head hyperpolarizes to the bud, and forms bright aggregates. This effect can be seen even when the first coiled-coil (the coiled-coil region is predicted to have two CC) is deleted. However, when both coiled-coils are lost,

staining of the tail or MBD domain itself is all diffuse, indicating that just Myo2p binding is not enough. These truncations also have different expression levels, which will be covered in detail in the next chapter. In summary, Smy1p localization seems to be more complicated than we expected, with different domains contributing in different ways.

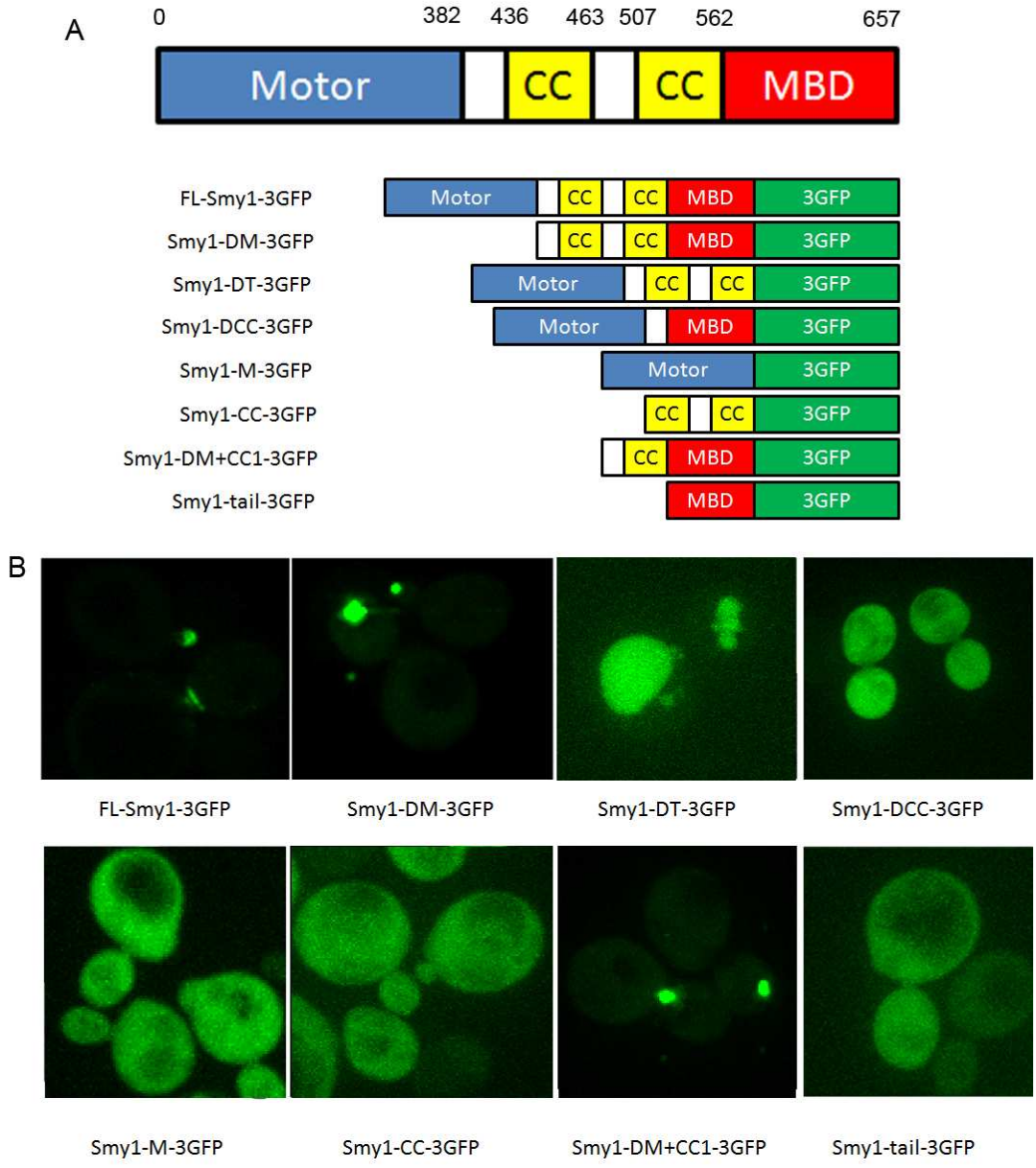


Fig 2.3 Smy1p truncations localize in various ways.
(A) Schematics of three domains of Smy1p: Motor-motor or head domain,

CC-coiled-coil region, MBD-Myo2p binding domain or tail. Truncations were constructed according to the map, expressed under its own promotor, tagged with 3GFP
(B) Smy1 truncations tagged with 3GFP visualized in WT cells at RT.

2.4 Smy1p distribution by cell fractionation

To further support my imaging data, cell fractionation experiments and western blot analysis were employed. The idea is that, since Smy1p localization is lost in *myo2-14* and *myo2-16* cells, maybe I can quantify this effect by monitoring the distribution of the protein on different membrane components. Although the result was not as expected, I feel it necessary to put these data in the thesis because I spent a lot of time in refining the system.

In this experiment, I tagged endogenous Smy1p with 3HA using the Longtine cassette (Longtine et al., 1998) in WT, *myo2-14* and *myo2-16* cells, because we didn't have Smy1p antibody at that time. After fractionation, theplasma membrane, ER and other large membrane compartments precipitate in the "P13" fraction, while "P100" mainly represents secretory vesicles. Sec4p was shown to distribute almost equally between these two fractions (Goud and Salminen, 1998), which was confirmed in my experiments. In addition, this localization can be disrupted by adding 1% Triton X-100. However, although Smy1p distribution is very similar to Sec4p, it remains in the "P100" fraction after 1% Triton X-100 is added (Fig 2.4). No obvious difference was found between the three strains used. In conclusion, this experiment is hard to

interpret since the fractionation is quite rough.

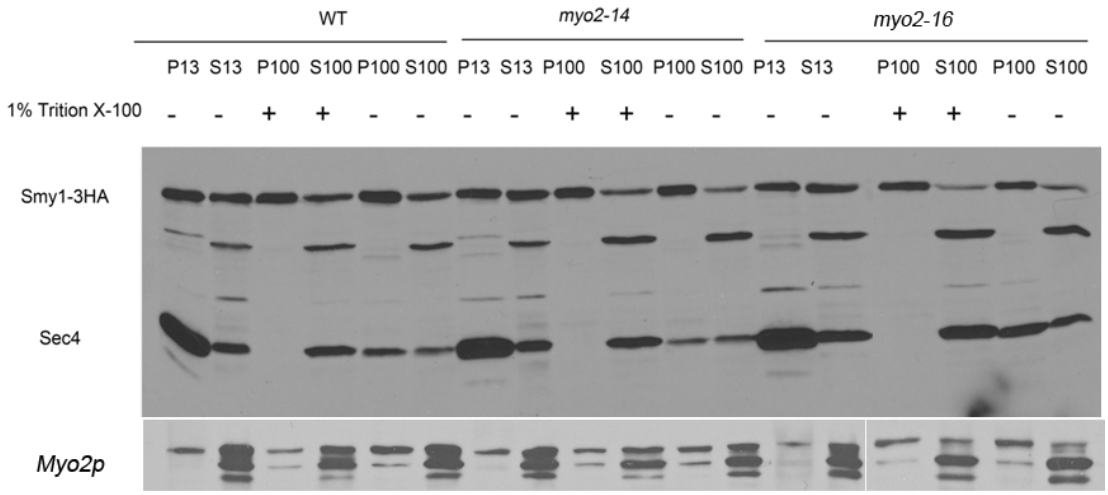


Fig 2.4 Smy1p stays to membrane fractions.
This Western was done by blotting with Myo2p antibody and then stripped to blot with anti-HA and anti-HA antibody. The image were cut and put together because some lanes were empty due to loading mistake. Sec4p mainly distributes to membrane fractions while Myo2p doesn't. However, Smy1p stays in P100 fractions in all mutants and even after Triton X-100 was added.

Conclusion and Discussion

In this chapter, I mainly investigated how Smy1p localization is achieved. The Trybus group proposed an electrostatic tether model, claiming Smy1p binds to secretory vesicles rather than Myo2p. My data clearly shows that Myo2p binding is necessary for Smy1p localization, because when this binding is lost, as in *myo2-14* and *myo2-24*, or weakened, as in *myo2-16*, Smy1p is mislocalized. However, this interaction is not sufficient either, due to the fact that in *myo2-12* and *myo2-16*, Myo2p is polarized but Smy1p is not. Besides that, I also studied Smy1p localization in *sec2-56* and *sec4-8* cells, which have depolarized Myo2p,

Smy1p and vesicles at the restrictive temperature. Because no good secretory vesicle marker was available here (GFP-Snc1p and RFP-Snc2p are both too dim), I couldn't tell if Smy1p and Myo2p stayed on secretory vesicles. However, by careful studying the fluorescence of Myo2p-3GFP and Smy1p-3mcherry, I didn't see any dots or blobs, but only diffuse staining. I would infer that Myo2p and Smy1p are both in the cytosol under these conditions. To further confirm that, immuno-fluorescence microscopy using anti-Sec4p and anti-Smy1p antibody is needed.

Taken together, Smy1p binds to secretory vesicles through binding to Myo2p, but this interaction cannot be maintained when Myo2p is detached, presumably because Smy1p also interacts with something else on secretory vesicle.

I also tried to determine the localization-determining domain of Smy1p, but the result was quite complicated. There seems to be a balance between the head and the tail domain, because without the tail the protein is degraded, while without the head the protein is hyperpolarized and aggregated. The coiled-coil (CC) regions are also very important since the head or tail by itself doesn't localize in the same way as when combined with CC. I will talk more about these truncations in the following chapter. I believe in these findings lies the molecular mechanism of Smy1p function. Further investigation is definitely needed.

CHAPTER 3: SMY1P EXPRESSION LEVEL

Introduction

For decades, Smy1p research has been focused on its genetic and physical interaction, as well as its localization. However, the molecular mechanism how this protein functions remains elusive. An interesting observation from my localization experiment of Smy1p truncations was that they all localizes in different ways. For example, Smy1p without the motor domain (Smy1DM) tagged with 3GFP forms extremely bright spots, while the fluorescence of Smy1p without the tail domain (Smy1DT) tagged with 3GFP almost cannot be seen (mostly in vacuole). This phenomenon inspired me to ask whether they have different expression levels. In addition, how is this expression level controlled?

Material and Method

Molecular cloning and vectors

Cloning techniques are the same as described in Chapter 2. Vectors used include pHL-Smy1-truncation-3GFP series, pRS415 prom HA-Smy1-6his, pRS415 prom UBI-R-HA-Smy1-6his, pRS415 prom UBI-M-HA-Smy1-6his, (prom is a weakened GAL promotor), pE-SUMO-Smy1 and the Longtine 3HA plasmid.

Immunoblots

Cells were grown to mid-log phase in 5ml liquid culture and the OD_{600}

was measured. After that, I spun down the cells at 1,000 rpm and washed them once with distilled water. Then the cells were transferred to 1.5ml tubes and resuspended in ice chilled 1X SDS loading buffer (40ul for each OD unit, i.e. 200ul for 5ml OD=1 culture), so that all samples are normalized to the same cell density. Following that, the same volume of ice chilled glass beads were added to each tube. The tubes were vortexed for 1min three times with 1min ice-cooling between each vortex. At last these samples were boiled for 5min, and spun down in clinical centrifuge at 13,000 rpm for 1min. The resulting supernatant was used for SDS-PAGE and western-blot.

Smy1p expression and purification

BL21 (DE3) competent bacteria was transformed with the pE-SUMO-Smy1 plasmid and selected on LB+Amp plates. A single colony was picked and grown in 5ml LB overnight, expanded to 1L the next day. When the OD_{600} reached ~0.5, I added 0.05% IPTG (500ul of 1000X stock) and induced overnight at 18℃. After 12 to 16h induction, cells were collected and resuspended in 50ml cold Binding Buffer (PBS pH7.5, 5% glycerol, 20mM imidazole) with protease inhibitor. Then the paste was sonicated (button-tip 5x30sec 50% duty cycle, output level 4-5) until the lysate was clear, and spun down at 12,000 rpm for 20min at 4℃.During the spin I equilibrated Cobl column (2ml Talon beads) with 10 column volume (cv) of Binding Buffer. Triton X-100 was added to 1% to the

supernatant, which was then carefully loaded into the Cobl column. Flow-through was collected for a gel sample. I washed the column with 20cv of Binding Buffer, then eluted with 3cv of Binding Buffer, followed by another elution (collect separately) with 3cv of Elution Buffer (binding buffer+300mM imidazole). Elution (elution1+2) was dialyzed in 2X 500mL of Binding Buffer overnight. Dialyzed protein was transferred to falcon tube with ~35-60ul of Ulp1 added per 500mL culture, incubated at 30°C for 40min to cleave SUMO tag. Then I poured 1ml Cobl column and equilibrated it with 10mL Binding Buffer. Cleaved protein was poured over the column and flow-through was collected. At last, I washed the beads with 1mL Binding Buffer and combined the two elutes. Protein was concentrated with 20% glycerol added, and frozen at -80℃.

Yeast synchronization

I used the α-factor release protocol (L. Breeden et al., 1997) to synchronize the yeast cells. Basically, cells were grown in 5ml liquid medium to early-log phase (OD_{600}=0.2~0.3). α-factor was added to 1X (200X stock is 1mg/ml). After 2h, I looked at the cells under light microscope to make sure they are arrested as round cells (G-1). Then, I washed the cells with the same volume of water for three times and resuspended them in the same volume of medium to resume growth. 500ul samples were taken every 15min for 2h. Cells would remain synchronized for ~2 generations.

Results

3.1 Smy1p protein level is cell-cycle independent

The first question we asked is whether the Smy1p protein level changes according to cell cycle. Here, I took advantage of the WT strain in which endogenous Smy1 is tagged with 3HA I made previously. Then I used α-factor release to synchronize the cells, and confirmed the effect by microscopy (Fig 3.1A). After that, Smy1p protein level was monitored every 15 min for 2.5h by WB with anti-HA antibody. The result showed that Smy1 level is cell-cycle independent (Fig 3.1B)

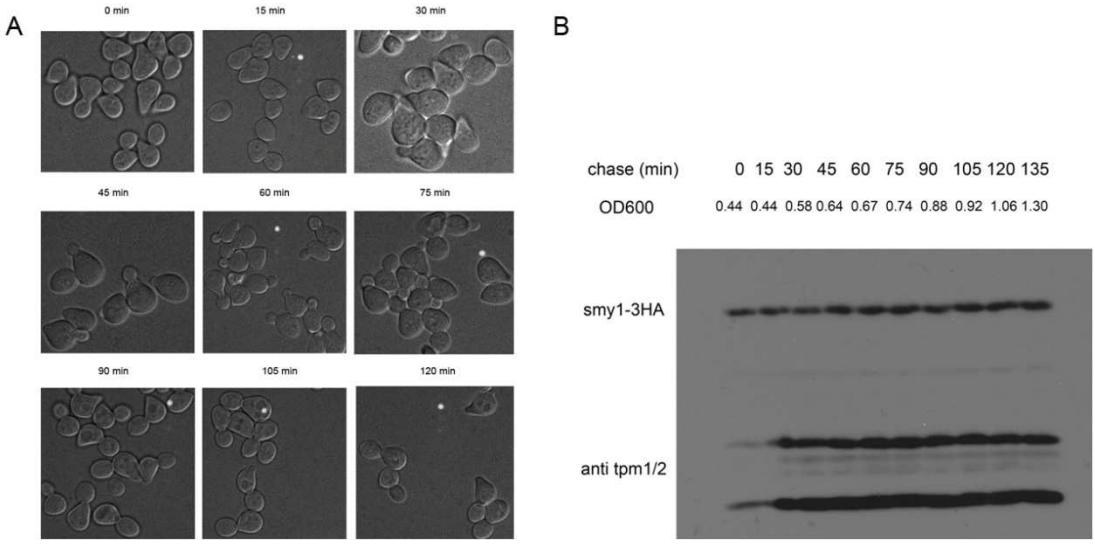


Fig 3.1 Smy1p level is cell-cycle independent.

(A) Cells were synchronized after α-factor release, and gradually went through the cell cycle in 2h

(B) Smy1p level were monitored by anti-HA WB, which doesn't change according to cell cycle. Tpm1/2 was used as loading control here.

3.2 Smy1p truncations have different expression levels

As mentioned in the introduction of this chapter, I started to pay attention to the expression level of different truncations shortly after the

imaging experiment. I used anti-GFP WB since they were all tagged with 3GFP and expressed under the native *SMY1* promotor. The result is quite interesting (Fig 3.2A). As you can see, full-length Smy1p expresses at a rather low level. When the motor domain is deleted, the expression level suddenly rose more than 10 times (DM, DM+cc1 and Tail), which explains why the staining of these proteins were so bright. When the tail domain is truncated, the expression dropped to an extremely low level (DT), which is dependent on the CC domain because the motor domain itself could still be detected. Consistent with the observations, Smy1DT-3GFP was extreme dim and mostly found in the vacuole, probably undergoing degradation. The CC region itself seems to be not so important because the protein level remain almost the same as FL when it is deleted.

To further investigate the regulation of Smy1p expression level, I also chased the protein by expressing it under GAL promotor and shutting down the expression by shifting to glucose medium. WT cells carrying the pRS415 prom Smy1-6his plasmid, which expresses Smy1p-6his under a weakened GAL promotor, were grown in Sraff+0.5% galactose medium overnight to mid-log phase. Then cells were aliquoted into two tubes, washed once with water, resuspended either in the same medium or in glucose medium. Smy1p level stays almost the same in the 2h chase. Therefore, Smy1p doesn't undergo quick turn-over (Fig 3.2B).

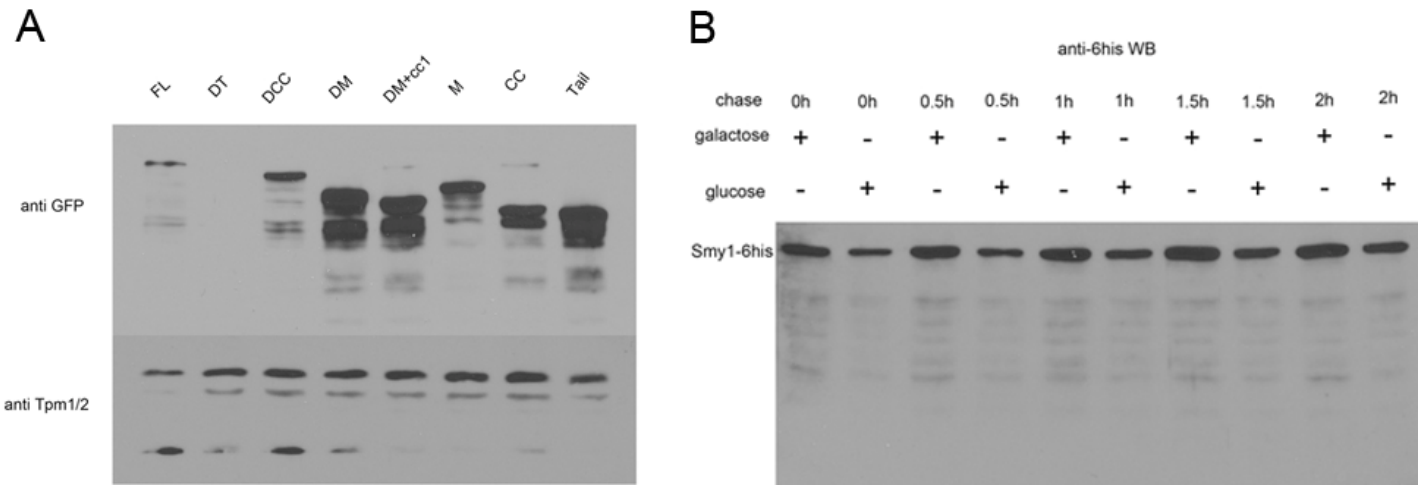


Fig 3.2 Smy1p truncation expression level and protein turn-over

(A) Cells expressing different Smy1p truncations were subjected to anti-GFP WB. Tpm1/2 is loading control. FL:full-length Smy1p. DT: Smy1p without tail domain. DCC: Smy1p without coiled-coil region. DM: Smy1p without mothr domain. DM+CC1: Smy1p without motor and first CC. M: Motor domain. CC coiled-coil region. Tail: tail domain. These constructs were all tagged with 3GFP.

(B) Same amount of cells were loaded to each lane. Western blots was performed using anti-6his antibody..

3.3 Smy1p is subject to post-translational modification

Smy1p protein is very curious not only because of the complicated expression level regulation, but also due to its unexpected behavior in another experiment, which will be covered in detail in the next chapter. Luckily, through an accidental experiment I solved this problem. In this experiment, WT cells were transformed with pRS415 prom HA-smy1-6his, pRS415 prom UBI-M-HA-smy1-6his or pRS415 prom UBI-R-HA-smy1-6his. These plasmids express HA-Smy1p-6his or N-terminal ubiquitin tagged HA-Smy1p-6his under a weakened GAL promotor. The ubiquitin tag here is used to expose the first amino acid of the fusion protein, which is

either methionine (M) or arginine (R). M is supposed to make this protein very stable and R would induce degradation (Park et al., 1992). I grew the cells in SRaff-Leu medium until mid-log phase and then induced the expression with 2% galactose for 4h. WB using anti-HA and anti-6his antibody were performed. Surprisingly, I can only detect the full-length protein with anti-6his antibody but not anti-HA antibody. Besides, I could see two bands for M construct but only one band for R construct (Fig 3.3). Therefore, the beginning few amino acids of Smy1p head are probably cleaved in the cell.

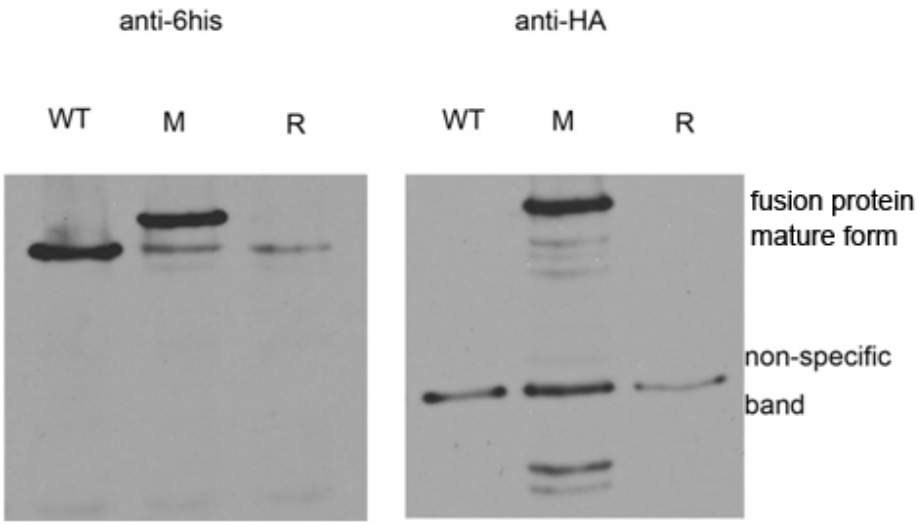


Fig 3.3 Smy1p undergoes post-translational modification
WT: HA-Smy1p-6his. M: UBI-M-HA-Smy1p-6his. R: UBI-R-HA-Smy1p-6his. The upper band is the fusion protein. The lower band is the mature form Smy1p-6his.

3.4 Smy1p antibody generation

Generating a Smy1p antibody is very important for the following research in the lab. So I expressed his-SUMO-Smy1p in bacteria and purified the untagged Smy1p according to the protocol in the material

and methods. To enhance the efficiency I repeated passing the flow-through, eluting the protein and regenerating the column for 3 times. Each time the protein able to bind to column decreased. At last I combined the three elutes and digested the His-sumo-Smy1p protein with Ulp1p. A SDS-PAGE was used to confirm the success of purification. You can see the digested untagged Smy1p is indeed a little bit smaller than the tagged one (Fig 3.4A). After that, I did a gel prep to further purify the protein and got 1ml Smy1p protein (~0.8mg/ml) for antibody generation (Fig 3.4B).

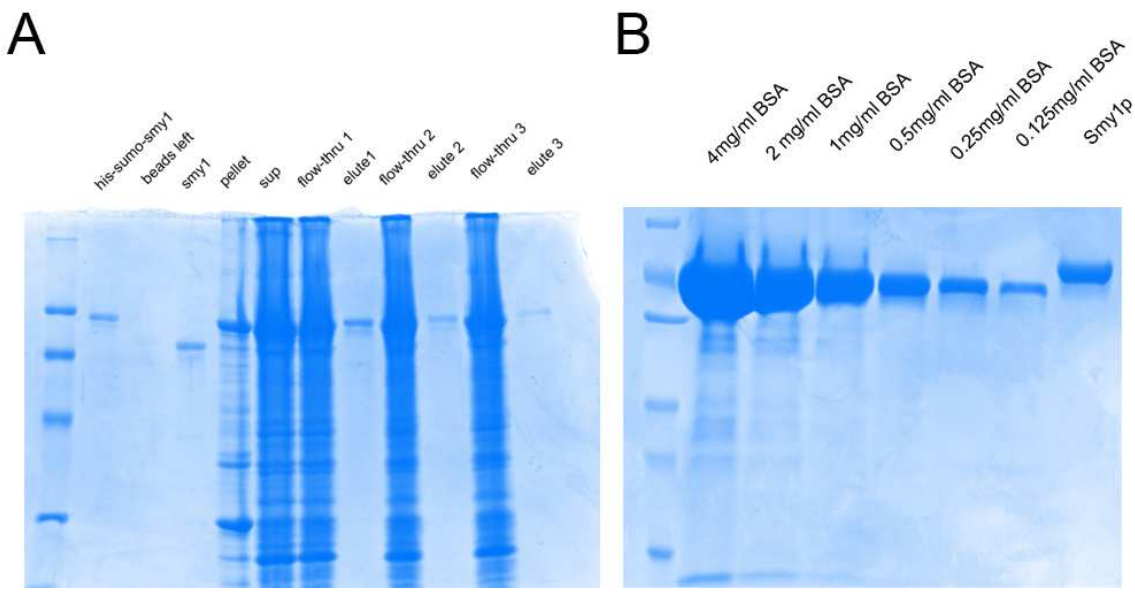


Fig 3.4 Smy1p purification
(A) 6his-SUMO-Smy1p was purified using the Cobl column.
(B) After the gel prep, I measured the concentration of Smy1p using BSA as standard. The Smy1p band is approximately 0.8 mg/ml.

Conclusion and Discussion

In this chapter, I started my investigation of Smy1p expression level by

chasing it through the cell cycle. Some adaptor proteins like Vac17p, part of the vacuole receptor for Myo2p, are tightly controlled according to cell cycle because the transport must happen in a timely and orderly way. Unfortunately, Smy1p level doesn't change with cell cycle, which is reasonable, because polarized secretion is constantly needed, unlike vacuole segregation.

Then how is Smy1p level controlled? Interestingly, different domains seem to have different effects. The tail domain stabilizes this protein, for the tail itself expresses at a high level. In contrast, the head domain antagonizes the tail, making the protein very unstable. However, only when the head or the tail is combined to the coiled-coil region could the effect be exaggerated, because Smy1p without its head domain forms aggregates in the bud while Smy1p without tail is quickly degraded in vacuole. Therefore, there seems to be an inner balance for Smy1p to make the choice of being degraded or to accumulate. And this balance may be very important for us to understand Smy1p function, as to how Smy1p is loaded onto secretory vesicles and how it is recycled back to the mother cell.

What's more, Smy1p seems to be post-translational modified, with the head domain clipped, although the mature protein is quite stable. To verify this, N-terminal GFP tagged Smy1p can be used and the fluorescence can be monitored. If this modification does happen *in vivo*,

the GFP signal should be diffuse. Furthermore, where this cleavage occurs and why it is needed is still unclear. One easy way to answer this question is to purify Smy1p-6his from yeast cells using Cobl column and send the protein for mass-spec, in order to determine the exact cleavage site. Once the site is known, mutants with a disrupted cleavage site can be generated, whose function and localization would be interesting for investigators.

CHAPTER 4:SMY1P DEPLETION IN DIFFERENT MUTANTS

Introduction

Genetic approaches have been playing an important role in our research for a long time. Budding yeast is very useful and convenient for genetic study, because it has a haploid and a diploid form. As haploid, the genome can easily be modified and the effect of the modification can readily be detected. Through mating and tetrad-dissection, different mutations can be combined to study synthetic effects, which would provide us with hints of the protein's function.

Previous research has clearly put Smy1p in a genetic interaction network, which is shown in Table 1 (adopted from SGD). As you can see, Smy1p seems to be involved in many processes, in which three aspects are especially prominent. First, Smy1p interacts with various genes regulating actin cytoskeleton assembly, including *BEM1, BEM2, BUD14, CLA4, BNR1, BNI1* and *PFY1*. From this perspective, as stated previously, the Goode group proposed Smy1p to be a Bnr1p suppressor. Second, Smy1p seems to also functions in cytokinesis, because it has synthetic effects with *BCK1, BUB1, CDC11, CDC12, CDC3, HOF1, MYO1* and *SHS1*, which are all related to bud neck formation. However, for now not much attention has been put in this area. Third, Smy1p is clearly implicated in late secretion, as *SEC2, SEC4, MYO2, CHS5* and *SNC2* all interact with it genetically. Smy1p also interacts with Myo2p physically, which is the only

interaction I have confirmed right now. One group claimed that Smy1p interacts with Bnr1p, but their two-hybrid data was not satisfactory (Kikyo et al., 1999). Sec2p was also claimed to interact with Smy1p (Zhang et al., 2009), but I couldn't verify that by Y2H myself. In this research, I am focusing on this aspect.

Table 3.1 Smy1p genetic interaction

Gene	Description	Interaction
ADH1	Alcohol dehydrogenase	Negative (high-throughput)
AIM44	Protein of unknown function	Negative (high-throughput)
BCK1	MAPKKK acting in the protein kinase C signaling pathway	Synthetic Rescue (manual currated) Negative (high-throughput)
BEM1	Protein containing SH3-domains	Negative (high-throughput)
BEM2	Rho GTPase activating protein (RhoGAP)	Synthetic Lethality
BNI1	Formin	*smy1 bud14 bni1* is synthetic lethal at high temperature
BNI4	Targeting subunit for Glc7p protein phosphatase	Negative (high-throughput)
BNR1	Formin	*smy1 bud14 bnr1* is synthetic rescue at high temperature
BUB1	Protein kinase involved in the cell cycle checkpoint into anaphase	Synthetic Lethality (high-throughput)
BUD14	Protein involved in bud-site selection	Synthetic Growth Defect
CDC11	Component of the septin ring that is required for cytokinesis	Negative (high-throughput)
CDC12	Component of the septin ring that is required for cytokinesis	Negative (high-throughput)
CDC3	Component of the septin ring that is required for cytokinesis	Negative (high-throughput)
CDC34	E2 enzyme and catalytic subunit of SCF ubiquitin ligase complex	Synthetic Lethality
CHS3	Chitin synthase III	Synthetic Growth Defect (high-throughput)
CHS5	Component of the exomer complex	Synthetic Growth Defect (high-throughput)
CHS7	Protein of unknown function	Synthetic Lethality (high-throughput)

CLA4	Cdc42p-activated signal transducing kinase of the PAK family	Synthetic Lethality (high-throughput)
CMD1	Calmodulin	Negative with *cmd1* mutant (high-throughput)
DRS2	TGN aminophospholipid translocase (flippase)	Synthetic Growth Defect (high-throughput)
DUN1	Cell-cycle checkpoint serine-threonine kinase	Negative (high-throughput)
ELM1	Serine/threonine protein kinase that regulates cellular morphogenesis	Negative (high-throughput)
GIM4	Subunit of the heterohexameric cochaperone prefoldin complex	Negative (high-throughput)
HOF1	Bud neck-localized, SH3 domain-containing protein required for cytokinesis	Negative (high-throughput)
ILM1	Protein of unknown function	Negative (high-throughput)
KIN3	Nonessential serine/threonine protein kinase	Negative (high-throughput)
MNN10	Subunit of a Golgi mannosyltransferase complex	Negative (high-throughput)
MYO1	Type II myosin heavy chain	Synthetic Growth Defect (manually curated)
MYO2	Myosin V	Synthetic Lethality and Rescue (manually curated)
PFY1	Profilin	Dosage Rescue
PHO85	Cyclin-dependent kinase	Synthetic Growth Defect (high-throughput)
SEC2	GEF for Sec4p	Synthetic Lethality
SEC4	Small GTPase	Synthetic Lethality
SHS1	Component of the septin ring that is required for cytokinesis	Negative (high-throughput)
SKT5	Activator of Chs3p	Negative (high-throughput)
SLA1	Cytoskeletal protein binding protein	Synthetic Growth Defect (high-throughput)
SLT2	Serine/threonine MAP kinase	Negative (high-throughput)
SNC2	v-SNARE	Negative (high-throughput)
SSD1	Translational repressor with a role in polar growth and wall integrity	Negative (high-throughput)
SWI3	Subunit of the SWI/SNF chromatin remodeling complex	Negative (high-throughput)
SWI4	Subunit of the SWI/SNF chromatin remodeling complex	Negative (high-throughput)
VPS4	AAA-ATPase involved in	Phenotypic Suppression

	multivesicular body protein sorting	(high-throughput)
YDJ1	Type I HSP40 co-chaperone involved in regulating HSP90 and HSP70	Negative (high-throughput)

Despite all these pieces of evidence, a fundamental problem is how do we interpret them and uncover the molecular mechanism behind it. As a way to investigate this problem, I conducted a series of experiment to observe the synthetic effect of Smy1p depletion in *myo2, sec2 or sec4* conditional mutants.

Material and Method

Molecular cloning and vector construction

Cloning techniques are as stated previously. Vectors used are: pRS415 prom (prom is a weakened GAL promotor), pRS415 prom UBI-M-smy1-6his, pRS415 prom UBI-R-smy1-6his. Yep351 SMY1 (2u), pRS316 SMY1, pRS416 GFP-SEC4, pRS416 GFP-SNC1, pRS303 sec2-56, pRS303 sec4-8

Gene knockout

Gene knockout was done using homologous recombination. Genomic DNA was extracted using the genomic DNA kit (Zymo Research) from the appropriate deletion collection strains. PCR was used to amplify the Kan cassette at the locus to be deleted. Then, the 2ul PCR product (~200ug/ul) was transformed into 5ml culture of yeast cells, using the Li-Ac protocol. Transformants were selected on YPD + G418 plates.

Colonies were picked and genomic DNA was extracted. A verifying PCR was done to make sure that the locus was successfully deleted.

Smy1p depletion

In this experiment, endogenous *SMY1* was deleted and Smy1p expression solely relies on the plasmid pRS prom UBI-R-smy1-6his. The cells were grown in Sraff-leu-ura +0.5% galactose medium overnight to reach mid-log phase. Then the cells were centrifuged at 1,000 rpm for 5min and washed once with distilled water. After that, they were resuspended in either the same medium or SD-leu-ura medium to grow for another 16h. At last, the cells were visualized under the fluorescence microscope.

Actin staining

Cells grown in 5ml liquid medium to the required density were fixed by adding 600ul 37% formaldehyde for 5min. Then the cells were pelleted and resuspended in 4.5ml 1XPBS+500ul 37% formaldehyde to fix for another 25 min. After that, cells were washed with 1XPBS three times to get rid of the formaldehyde (this is vital). Then they were transferred to 1.5ml tubes and resuspended in 200ul 1XPBS+20ul Alexa-568 phalloidin (1% Triton X-100 may be added), kept in dark for 1h with occasional vortexing. At last, these cells are washed four times with 1ml 1XPBS and subject to microscopy.

Results

4.1 Smy1p overexpression still suppresses *myo2-16* when either *BNR1* or *BNI1* is deleted

The Goode group claimed that Smy1p is a negative regulator of the formin Bnr1p, which localizes to bud neck. Indeed, Smy1p was shown to possibly be involved in actin cytoskeleton regulation. However, this model cannot explain why Smy1p overexpression, which presumably reduces the number and length of actin cables in the mother cell, could suppress *myo2* head and tail conditional mutants. Therefore, I set out to explore whether this phenotype is related to formins.

bnr1Δ myo2-16 and *bni1Δ myo2-16* double mutants were made as described in material and methods. In this experiment, *myo2-14*, *myo2-16*, *bnr1Δ myo2-16* and *bni1Δ myo2-16* were transformed with Yep351 *SMY1* (2u) or an empty vector. The growth of these strains was tested by a 5X serial dilution assay at 26□ and 32□. As you can see (Fig 4.1), temperature sensitivity (TS) of *myo2-14* cannot be suppressed by multi-copy *SMY1* while *myo2-16* can. When either *BNR1 or BNI1* was deleted in *myo2-16* cells, the TS phenotype could still be suppressed by overexpressing Smy1p. Therefore, Smy1p's function in late secretion is not related to formins.

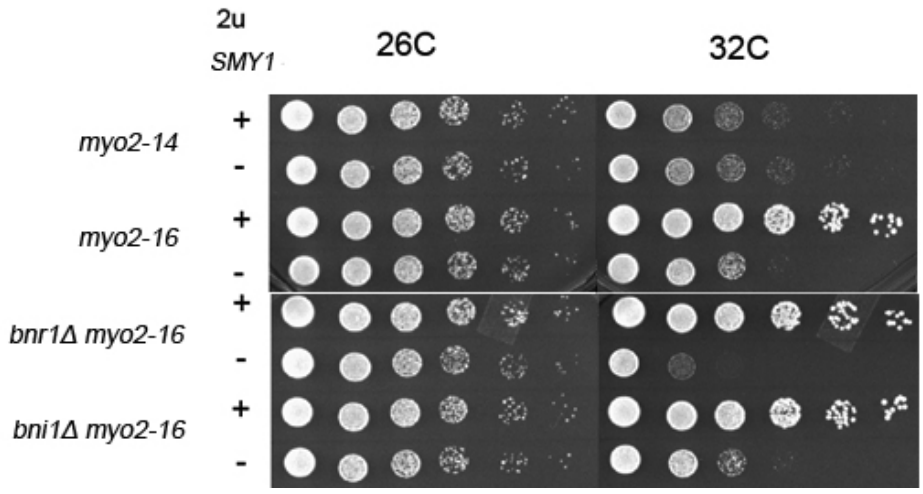


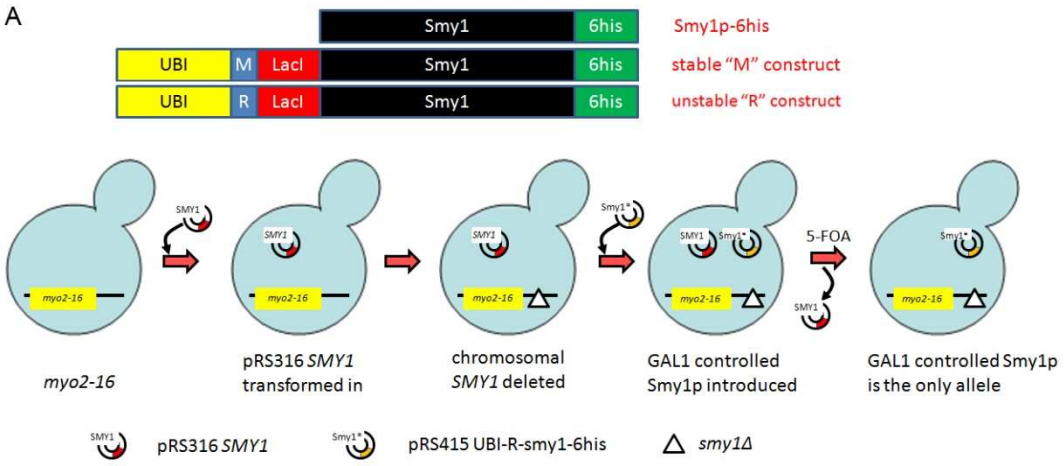
Fig 4.1 Smy1p overexpression still suppresses *myo2-16* temperature sensitivity when either Bnr1p or Bni1p is lost. *myo2-14* is used as a negative control here, which we knew cannot be suppressed by multi-copy *SMY1*.

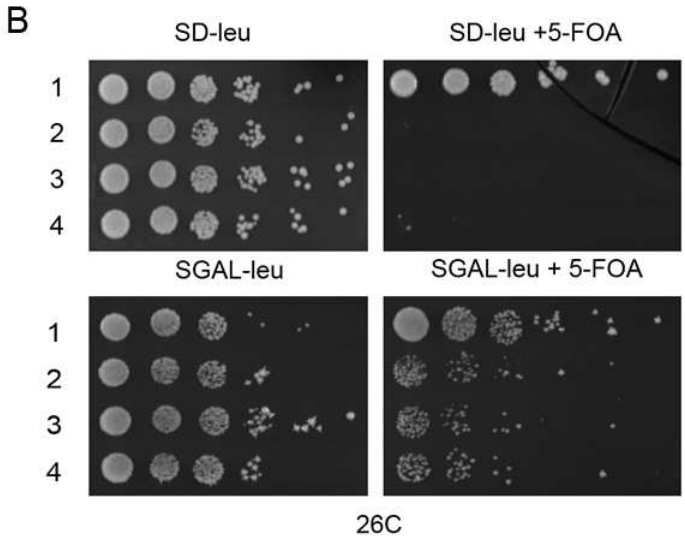
4.2 Smy1p depletion in *myo2-16* causes a block in secretion

myo2 tail conditional mutants generated in our lab were found to be synthetic lethal with *smyΔ* (Schott et al., 1999). Similarly, *SMY1* becomes essential in *sec2, sec4* and other conditional mutants. These genetic data prompted us to investigate Smy1p function in polarized secretion. However, why these genetic interactions exist or how these double mutants lost the ability to survive is very curious, and the phenotype would be even more informative for our research. The first idea may be to generate some temperature sensitive (TS) mutants, but *myo2, sec2 and sec4* mutants are already TS. As a result, I decided to try to solve this problem by depleting Smy1p and observing the phenotype.

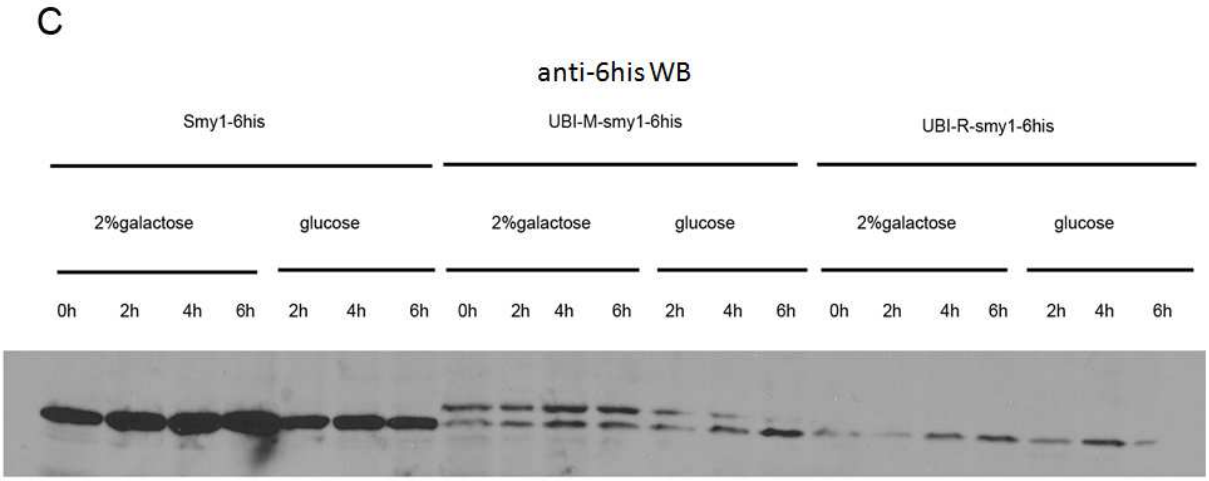
The method (Park et al., 1992) is to replace the endogenous *SMY1* allele with an easily degraded *SMY1* copy controlled by the GAL promotor (Fig

4.2A). Upon shifting to glucose medium, the expression is shut down and the protein is quickly degraded, so that the synthetic effect can be monitored. I successfully constructed all the plasmids needed and verified that the new Smy1p allele is functional (Fig 4.2B). However, this allele was not quickly degraded as expected, but maintained a quite low level (Fig 4.2C). The reason for that was stated in the previous chapter (Fig 3.3). N-terminal of Smy1p is processed in the cell, which also takes off the ubiquitin tag that destabilizes the protein. This modification likely happens slowly or the efficiency was decreased due to the N-terminal tag, because most of the protein is quickly degraded, with only a small amount of mature form left. This small portion of Smy1p is enough to support cell growth, and it is stable for at least 6h. Despite that, I decided to try this method by waiting for 16h for the protein to be naturally diluted. The result was quite interesting (Fig 4.2D). When Smy1p is depleted in *myo2-16 smy1Δ* cells, the cell accumulates secretory vesicles in the mother cell, especially at the bud neck. This has also been seen in other mutants like *myo2-12* and *myo2-16* at high temperature, indicating a secretion block. As a control, actin staining was included to exclude the possibility that the secretion block was caused by disrupted actin cytoskeleton.





1. *myo2-16* +pRS415 GAL-SMY1-6his+pRS316 *SMY1*
2. *myo2-16 smy1Δ*+pRS415 GAL1-SMY1-6his+pRS316 *SMY1*
3. *myo2-16 smy1Δ*+pRS415 GAL1-UBI-R-SMY1-6his+pRS316 *SMY1*
4. *myo2-16 smy1Δ*+pRS415 GAL1-UBI-M-SMY1-6his+pRS316 *SMY1*



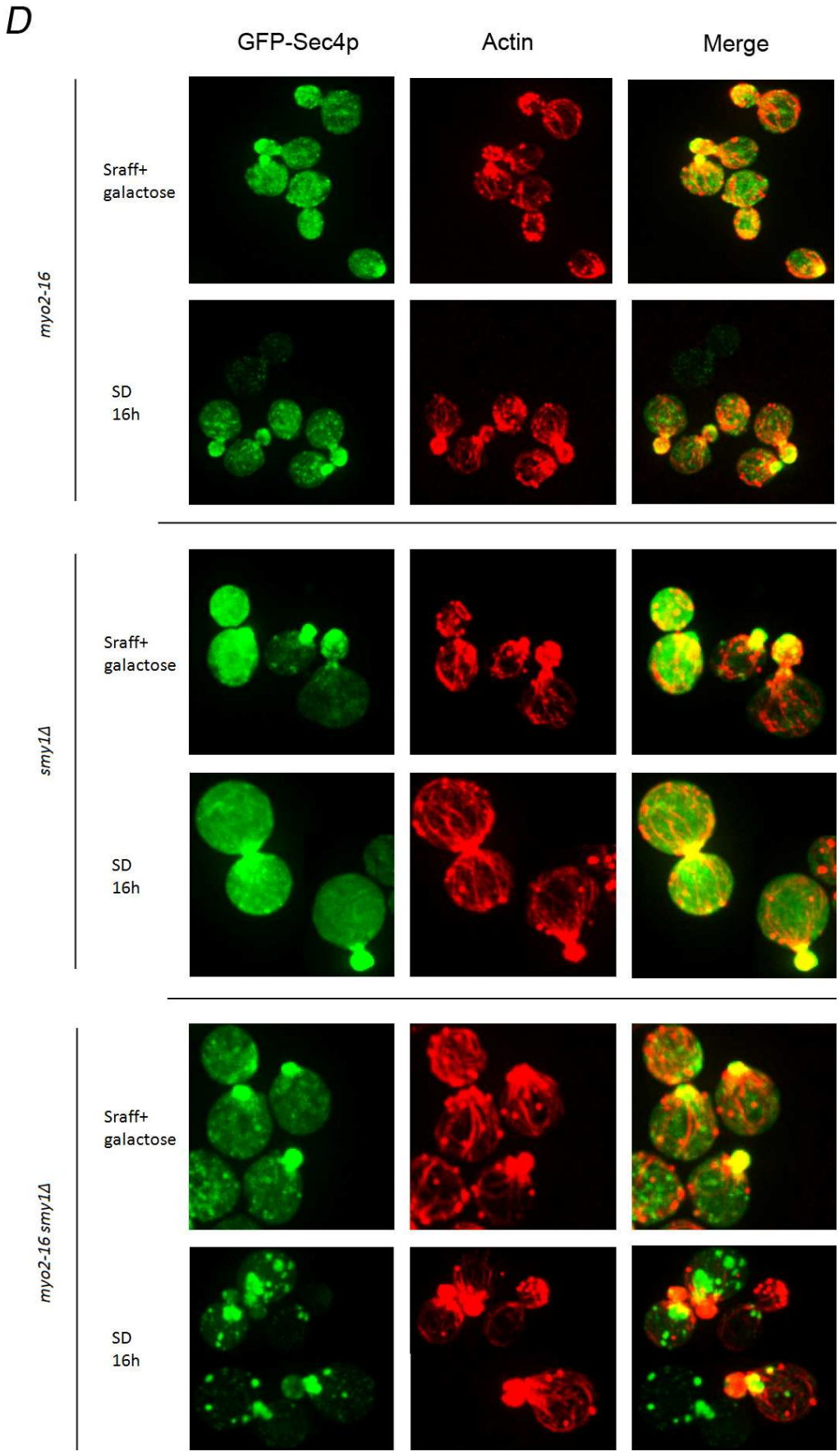

D
GFP-Sec4p
Actin
Merge
myo2-16
Sraff+ galactose
SD 16h
smy1Δ
Sraff+ galactose
SD 16h
myo2-16 smy1Δ
Sraff+ galactose
SD 16h

Fig 4.2 Smy1p depletion in *myo2-16*

(A) Schematics of plasmids used and strain generation Smy1p was N-terminal tagged with first an ubiquitin, followed by a methionine or arginine, then a fragment from bacteria $LacI_q$. When the protein is expressed, it will soon be deubiquitinated so the first amino acid after ubiquitin will be exposed. Arginine will destabilize the protein while methionine does the opposite. The LacI fragment contains two lysines which can be ubiquitinated. Together, the R-construct will be sent to the proteasome for degradation quickly.

(B) UBI-R-smy1-6his is able to replace the endogenous *SMY1* in *myo2-16.* 1 is a positive control since the endogenous *SMY1* is intact. 2, 3 and 4 are expressing different Smy1p constructs, which could complement the *smy1* deletion when induced on SGAL plates, and suppress the synthetic lethality.

(C) Smy1p construct degradation. WT cells expressing different Smy1p constructs were grown in Sraff+2%galactose medium overnight and shifted to either the same medium or SD medium. Protein levels were monitored by anti-6his WB for 6h. Due to the N-terminal processing of Smy1p, you can see two bands for UBI-M-smy1-6his. UBI-R-smy1-6his has only one band and expresses at very low level, but is stable for 6h.

(D) Smy1p depletion in *myo2-16* causes a secretion block. After UBI-R-smy1-6his expression is shut down for 16h, *myo2-16 smy1Δ* accumulate secretory vesicles in the mother cell, especially at the bud neck, while actin cables are intact. GFP-sec4 was used as vesicle marker.

I have also planned to conduct the same experiment in *sec2-56*, *sec2-59* and *sec4-8* cells. I constructed pRS303 *sec2-56*, pRS303 *sec2-59* and pRS303 *sec4-8* plasmids, and transformed them into WT BY strain to generate the *sec2-56, sec2-59* and *sec4-8* mutants in the BY background, because BY strain is believed to be healthy and it is GAL+. I have also sequenced the strains and verified the temperature sensitivity phenotype. However, I didn't have time to carefully refine the system.

Conclusion and Discussion

In previous research, genetic approaches were widely employed and provided us with plenty of hints to understand the function of proteins

encoded by different genes. High-throughput methods like SGA largely expanded our ability to study genetic networks, although not all the data are reliable. Smy1p was shown to be involved in a lot of processes related to polarity establishment. Actin cytoskeleton assembly, in which formins play an important role, was one area that Smy1p could influence. However, whether this activity is related to its function in secretion was unclear. Through my experiments, we found that deleting either formin doesn't affect Smy1p's suppression of the *myo2* conditional mutant *myo2-16*. More conditional mutants may be used to further support the idea that Smy1p has a separate function in secretion.

What's more important, the result from the Smy1p depletion experiment is the first direct evidence that Smy1p is really related to secretion. Although the construct I used didn't work as expected, a 16h shift still gave us a very strong phenotype. These accumulated secretory vesicles suggest that when Smy1p is lost, mutant Myo2p cannot transport its cargo. Therefore, Smy1p seems to play an accessory role that helps Myo2p to move vesicles, but we still don't know how Smy1p functions. One possibility is that, without Smy1p, mutant Myo2p cannot stay attached to secretory vesicles due to weakened interaction with Sec4p. It would be very interesting to look at Myo2p localization when Smy1p is depleted. I did try to do this using immuno-fluorescence with anti-Myo2p tail antibody, but the experiment didn't work well. Besides

that, the same experiment can also be tried in *sec2* and *sec4* conditional mutants. The fundamental problem of these mutants is that Sec4p stays in the GDP-bound form most of the times, reducing its ability to recruit and activate Myo2p. With Smy1p, this is possible at permissive temperature. Without Smy1p, this initiation step probably cannot happen. That's why *sec2-56* and *sec4-8* are synthetic lethal with *smy1Δ*. If we can look at secretory vesicle and Myo2p localization in these conditions, maybe a new model of Smy1p function in late secretion can be established.

CHAPTER 5: *SMY1* MUTANTS IN *MYO2* SENSITIZED BACKGROUND

Introduction

Smy1p is a non-essential gene, which means any mutation of Smy1p would hardly have any effect on the cell. However, to study molecular mechanism of a protein's function, mutational analysis is indispensable. Therefore, one can only try to study the synthetic effect of *smy1* conditional mutants in a strain where *SMY1* is essential. We call this kind of strain a "sensitized strain". There are a lot of sensitized strains for Smy1p, as shown in Table 3.1, but most of them are not usable because the strains are already temperature sensitive. So we set out to generate non-temperature-sensitive sensitized strains and then to generate *smy1* conditional mutants in those backgrounds.

Material and Method

Vectors

p22 *SMY1*, pRS316 *SMY1,* pRS303 *myo2* tail mutant library, pRS304 *smy1* mutant library, pGADT7 Myo2-cctail of 17 *myo2* tail mutants I identified, pGADT7 WT Myo2-cctail, pGADT7 vector, pAS2 Smy1, pBridge Sec4ΔC, pBridge Sec4ΔC +NLS-Smy1, pRS416 GFP-sec4, pRS316 *MYO2* and Yep351 *SMY1.*

Synthetic lethal screen

This experiment was performed as previously described (Drain and Schimmel, 1988). I transformed *smy1Δ ade2Δ ade3Δ* strain (CUY30 background) with p22 *SMY1*, which carries *ADE3* gene and makes the colony red. This strain will sector on 1/5ADE SC plates, because *SMY1* is not essential and when the plasmid is lost the colony becomes white. I grew the transformed strain to OD_{600}=0.8 in –His medium, so that all the cells carry the plasmid. Then I took 1ml of culture and added 30ul EMS (3% final), let the tube sit at RT for 30min with vortexing every 10min. After that I spun down the cells at 5,000 rpm for 5min in a clinical centrifuge, and washed the pellet with 1ml 12% sodium thiosulfate to inactivate EMS, followed by three washes with 1ml ddH_2O. At last, the cells were resuspended in 1ml H_2O and diluted 1:1000 in YPD. I spreaded 100ul diluted cells onto SC 1/5ADE, which were then incubated at 30□ for 2days and at 4□ for 3 days. Any colonies showing sectoring phenotype will be picked.

Error-prone PCR and library construction

The protocol for error-prone PCR is as described (Cadwell and Joyce, 1992). The key to get a low mutagenesis rate is to lower the manganese concentration. The buffer system I used is as follows:

	stock	final concentration	volume (ul)
template	60ng/ul	6ng/ul	15
F-primer	10uM	0.2uM	6
R-primer	10uM	0.2uM	6
dCTP	100mM	1mM	3
dTTP	100mM	1mM	3

dATP	10mM	0.2mM	6
dGTP	10mM	0.2mM	6
Taq PCR buffer	10X	1X	30
$MgCl_2$	25mM	2.5mM	30
$MnCl_2$ (add last)	20mM	0.15mM	2.25
Taq polymerase	5U/ul	0.05U/ul	3
Tween 20	1%	0.05%	15
BSA	10ug/ul (10X)	0.1ug/ul	30
ddH_2O			144.75
Total			300

After mixing the 300ul reaction, I aliquoted them into 6 tubes and ran a PCR at 50℃ for 10 cycles. The mutagenesis rate was about 0.67/1000nt. For *smy1* mutagenesis, I first constructed the pRS304 *SMY1* plasmid, which carries the WT *SMY1* gene and can be linearization by BamHI digestion. Then I used this plasmid as template, and F-smy1-inte as forward primer, R-smy1-inte3 as reverse primers in the error-prone PCR to generate the mutagenized *smy1* insert. Following that, to make a library, I digested the mutagenized *smy1* insert and the pRS304 *SMY1* plasmid with NotI and XhoI, and ligated them together. When transforming bacteria with the ligation reaction, I made sure more than 10,000 colonies were grown on LB+Amp plates. Then all the colonies were collected and cultured in 500ml LB+Amp liquid media for 2h at 37℃. At last, a maxi-prep was performed for these bacteria and the resulting plasmid would be a mutant library.

Mutant screen

All screens were done by manually picking the colonies and resuspending them in 150ul of ddH_2O in 96-well plates. Then, 5ul of the

mixture was spotted onto multiple plates to test different conditions. Strains that showed different growth were picked, streaked to single colonies and tested again. Only those showing the same phenotypes were kept and sequenced.

Results

5.1 Screen for *myo2* sensitized strains

To screen for sensitized strains, I chose to start with *myo2* mutants first, because Smy1p is tightly related to Myo2p function and we know Smy1p interacts with Myo2p both physically and genetically. To mutagenize *MYO2*, two methods were employed: EMS random mutagenesis and error-prone PCR mutagenesis.

The EMS random mutagenesis was done in my rotation, in a colony-sectoring screen (Koshland et al., 1985) searching for genes that are synthetic lethal with *smy1Δ*. The screen itself was not very successful since I only found three mutant strains. Fortunately, when I transformed another copy of *MYO2* into these strains, two of them could be suppressed, suggesting that there may be recessive mutations at the *MYO2* locus. Indeed, when I sequenced the *MYO2* gene, two myo2 *head* mutants were found. One of them, named *myo2-209*, has a single point mutation in the head domain (nt: 2090 C to T, aa: 697 T to I). The other one of them, called *myo2-247*, also has a single point mutation in the

head domain (nt: 247 G to A, aa: 83E to K). As for phenotype, both mutants are not sensitive to heat (viable at 37℃) but grow slowly. The doubling time of *myo2-209* is about 4h, while *myo2-247* doubles roughly in 3h.

Another way to generate sensitized strains is to use error-prone PCR and construct a mutant library, which will directly mutagenize the region of gene when transformed. Here I took advantage of an existing library of *myo2* tail mutants (from Dr. Irina Chernyakov). This mutant library is based on the pRS303 vector, which mutagenizes only the tail region (aa1093 to aa1574) of *MYO2* gene and linked it to a *HIS3* marker. I transformed this library into the *smy1Δ* + pRS316 *SMY1* strain (BY MATa), and got ~200 colonies/plate with 20 plates. After that, I manually picked ~2,500 colonies and spotted them onto –HIS and –HIS+5-FOA plates, incubated at 26℃ for 3 days. 33 candidates that didn't grow on –HIS +5-FOA plates were found, streaked to single colonies and tested again. At last, 17 mutants were left, from which genomic DNA were extracted and the smy1 locus was amplified by PCR then sequenced (Table5.1). All the mutants, except for *myo2-48*, are not temperature sensitive. I also cloned these mutants onto the pRS303 *MYO2* tail plasmid and transformed them back to the starting strain *smy1Δ* + pRS316 *SMY1* strain (BY MATa). For every transformation I picked 3 colonies and spotted them on –His and –His +5-FOA plates, to make sure that the

synthetic lethality can be repeated in at least one colony (Fig 5.1C).

Table 5.1 Mutations of myo2 tail sensitized strains

Bold: amino acids that cluster in the Rab binding site

Name	Mutations
myo2-41	**1462 I to T**
myo2-42	**1416 N to D** 1571 Q to H
myo2-43	**1446 L to P**
myo2-44	1310 L to Q 1475 I to S
myo2-45	1242 N to T **1448 V to A**
myo2-46	**1446 L to P**
myo2-47	**1347 F to S**
myo2-48	1295 K to E 1369 M to K **1453 I to N** 1478 Y to F
myo2-49	**1446 L to P** 1519 S to C
myo2-50	**1411 L to P** **1431 D to V**
myo2-51	**1444 K to M**
myo2-52	**1446 L to R**
myo2-53	1186 D to V **1416 N to Y**
myo2-54	**1346 I to T** 1491 I to T 1541 K to N
myo2-55	**1347 F to Y**
myo2-56	**1331 L to F** **1336 A to S**
myo2-57	1367 W to R **1415 Y to S** 1520 S to G

As you can see, some mutations have shown multiple times, like 1446 L to P, and they cluster mainly to the Rab binding sites (Fig 5.1A and B).

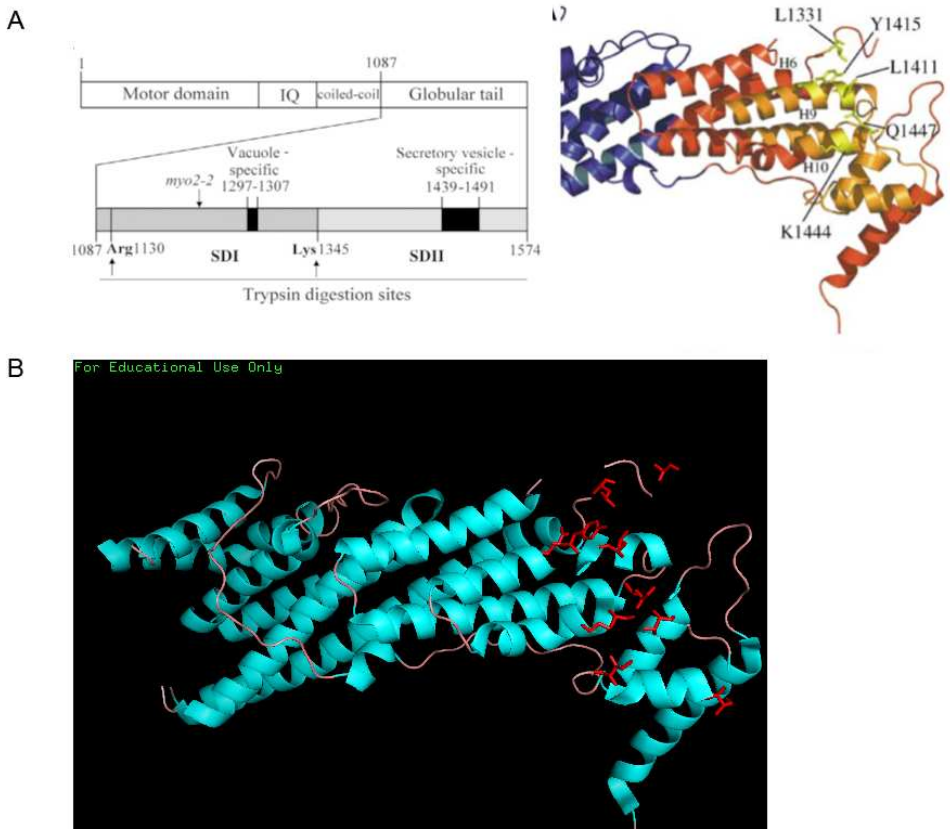

A
1
1087
Motor domain
IQ
coiled-coil
Globular tail
Vacuole - specific 1297-1307
myo2-2
Secretory vesicle - specific 1439-1491
1087 Arg1130
SDI
Lys1345
SDII
1574
Trypsin digestion sites
L1331
Y1415
H6
L1411
H9
Q1447
H10
K1444
B
For Educational Use Only
C
-His
-His+5-FOA
myo2-41
myo2-42
myo2-43
myo2-44
myo2-45
myo2-46
myo2-47
myo2-48
myo2-49
myo2-50
myo2-51
myo2-52
myo2-53
myo2-54
myo2-55
myo2-56
myo2-57

Figure 5.1 Mutations of *myo2* tail sensitized strains.

(A) For convenience of comparison, this figure was adopted from paper from Pashkova and Weisman in 2005 and 2006, showing the two subdomains of Myo2p tail and the important amino acids of the Rab binding site on the structure.

(B) Mutations of *myo2* tail sensitized strains were mapped onto the structure, with the ones on the Rab binding site shown in red. You can see clearly clustering of mutations in this region. Some mutations that map to subdomain-I were not shown, but could possibly be important as well.

(C) After *myo2* tail sensitized strains were identified, genomic DNA from themwas isolated. Using the genomic DNA as template, I cloned the *myo2* locus into the pRS303 *MYO2*-tail backbone and re-transformed them into *smy1Δ* + pRS316 *SMY1* strain (BY MATa). 3 single colonies of different sizes were picked from each transformation and spotted onto –His and –His+5-FOA plates to test the synthetic lethality. You can see at least one colony can grow on –His but not on –His+5-FOA plates.

Therefore, I tested the interaction of the mutant myo2-cctails with Smy1p and Sec4p respectively through Y2H. In this experiment, I cloned the mutant *myo2* cctails into the pGADT7 Myo2-cctail backbone to generate the pGADT7 *myo2* mutant-cctail series plasmids, which expresses AD-*myo2*-cctail protein in the nucleus. pAS2 Smy1, pBridge Sec4ΔC, pBridge Sec4ΔC +NLS-Smy1 plasmids were used to express the BD-Smy1p, BD- Sec4ΔC and BD- Sec4ΔC plus NLS-Smy1. The yeast two-hybrid experiment was done following the protocol described previously (*Material and Method*, Chapter 2). The results clearly tell us that most of the mutants have lost their interaction with Sec4p, while at the same time maintaining their interaction with Smy1p (Table 5.2). Unfortunately, expressing NLS-Smy1p from the pBridge Sec4ΔC+NLS-Smy1 plasmid (Made by Daniel Schott, the second multiple cloning site of pBridge carries a NLS-Smy1 construct) could not restore

the interaction between Sec4p and the mutant Myo2p cctails, although the expression was not verified due to lack of Smy1p antibody.

Table 5.2 Summary of Y2H interaction of myo2 tail mutants

These results are base on a 5X dilution assay. ++: grow very well in all dilutions; +/-: grow weakly but can still be seen in the first 3 dilutions; -: no growth
2DO: -Leu-Trp 3DO:-Leu-Trp-His 4DO:-Leu-Trp-His-Ade (very stringent)

AD-cctail	BD	2DO 26℃	3DO 26□	4DO 26℃	2DO 35℃	3DO 35℃
WT	Sec4p	++	++	-	++	++
	Sec4P+NLS-Smy1p	++	++	-	++	++
	Smy1p	++	++	++	+	++
Vec	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	-	-	++	-
myo2-12	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	++
myo2-14	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	-	-	++	-
myo2-16	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	-	++	+/-
myo2-41	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	++
myo2-42	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	++
myo2-43	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	++
myo2-44	Sec4p	++	+/-	-	++	-
	Sec4P+NLS-Smy1p	++	+/-	-	++	-
	Smy1p	++	++	++	++	++
	Sec4p	++	+/-	-	++	-

myo2-45	Sec4P+NLS-Smy1p	++	+/-	-	++	-
	Smy1p	++	++	-	+	-
myo2-46	Sec4p	++	+/-	-	++	-
	Sec4P+NLS-Smy1p	++	+/-	-	++	-
	Smy1p	++	++	++	++	++
myo2-47	Sec4p	++	++	-	++	++
	Sec4P+NLS-Smy1p	++	++	-	++	++
	Smy1p	++	++	++	+	++
myo2-48	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	-	-	++	-
myo2-49	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	++
myo2-50	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	++
myo2-51	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	+	++
myo2-52	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	++
myo2-53	Sec4p	++	-	-	++	-
	Sec4P+NLS-Smy1p	++	-	-	++	-
	Smy1p	++	++	++	++	-
myo2-54	Sec4p	++	+	-	++	++
	Sec4P+NLS-Smy1p	++	+	-	++	++
	Smy1p	++	++	++	++	+/-
myo2-55	Sec4p	++	++	-	++	++
	Sec4P+NLS-Smy1p	++	++	-	++	++
	Smy1p	++	++	++	++	+
myo2-56	Sec4p	++	++	-	++	++
	Sec4P+NLS-Smy1p	++	++	-	++	++
	Smy1p	++	++	++	++	++
myo2-57	Sec4p	++	+/-	-	++	-
	Sec4P+NLS-Smy1p	++	+/-	-	++	-
	Smy1p	++	++	++	+	-

5.2 Screen of *smy1* mutants

To screen for *smy1* mutants in the sensitized strains, a *smy1* mutant

library was constructed using error-prone PCR. This library was based on pRS304 vector and the whole *SMY1* gene was mutagenized. After linearization with BamHI and transformation into the cell, this mutagenized *smy1* will replace the endogenous allele through homologous recombination, and will be linked to a *TRP1* gene.

I started my screen in *myo2* head mutant *myo2-247,* which grows at a slightly slower rate than WT and is not temperature sensitive. The other head mutant *myo2-209* has a strong growth defect by itself and therefore was not chosen here, because it would be hard to interpret the phenotype of the double mutants if the contribution from *smy1* cannot be determined. Transforming pRS304 *smy1* into *myo2-247* gave me roughly 5,000 colonies. From them I picked 1,500 colonies and tested the growth at 26℃ and 37℃. Only two mutants were found to be TS, which are *myo2-247 smy1-15* and *myo2-247 smy1-17.* After that, genomic DNA was extracted and insertion was verified through a PCR reaction (Fig 5.2A). Then, both alleles were sequenced: *smy1-15* has a single point mutation in Smy1p head (269F to L); *smy1-17* has two point mutations in the head domain as well (117F to S and 121S to F). The latter is interesting because this region was homologous to other Kinesin's ATPase region (Lillie and Brown, 1992). At last, both of the mutations were transformed back to *myo2-247* cells to make sure that the TS phenotype was because of the synthetic effect of the two mutations.

Unfortunately, *myo2-247 smy1-15* didn't show the same heat sensitivity as before and was therefore abandoned.

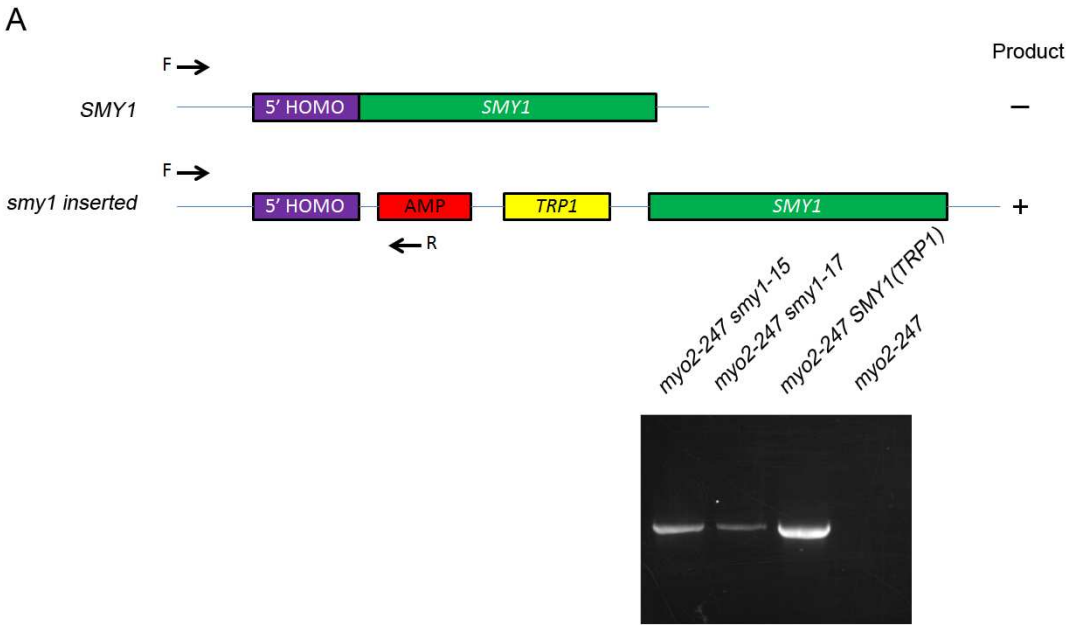


Fig 5.2 (A) smy1 insertion verification. One primer corresponds to the sequence before the 5' homologous region of *SMY1*, the other overlaps with sequence in the *AMP* gene. Product will only be seen when *smy1* is correctly inserted.

In addition, a screen in *myo2* tail sensitized strains was also performed. I picked 5 different sensitized strains, *myo2-41, myo2-43, myo2-47, myo2-51* and *myo2-57* because they all carry mutations near the Rab binding site. Similarly to the previous screen, I picked ~2,000 colonies from every background and screened for temperature-sensitive growth at 37℃. After careful selection and verification, 33 mutants were identified (Fig 5.2B). Through the same PCR reaction as in Figure 5.2A, they were all confirmed to have *smy1* insertion at the right locus. Sequencing of these mutants is still in progress, with part of the results shown in Table 5.3.

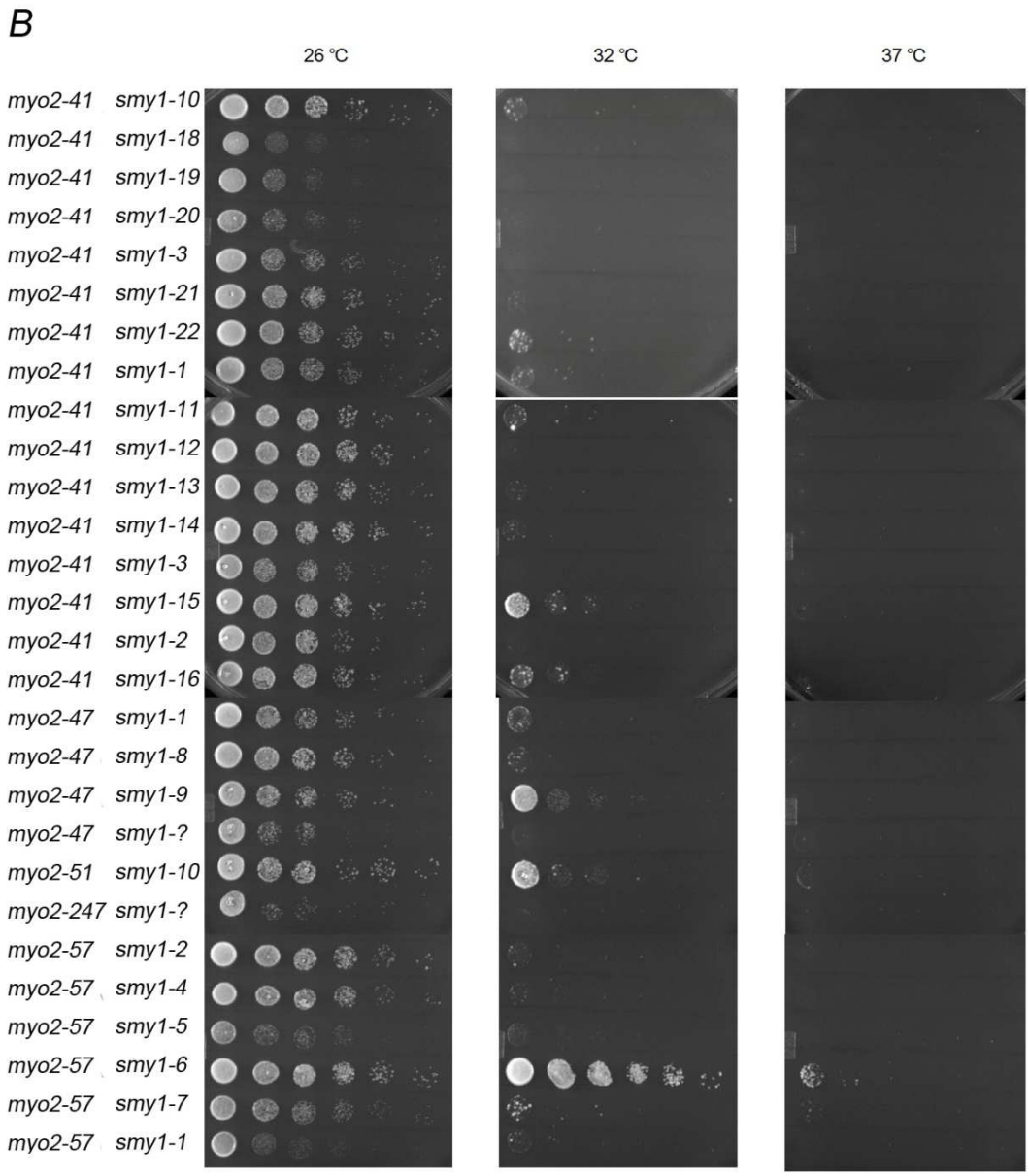


Figure 5.2 (B) smy1 mutants TS test. I got 16 mutants in *myo2-41* background, 4 mutants in *myo2-47* background, 1 mutant in *myo2-51* background and 6 mutants in *myo2-57* background. Unfortunately I didn't get any mutants in *myo2-43* background, although over 2,000 colonies were picked. Note that another *myo2-247 smy1* mutant is also put here, just for convenience. After I sequenced the *smy1* locus, I named these *smy1* alleles. Two of the mutants didn't have any mutation so I put a "?" as the allele name.

Table 5.3 *smy1* mutants sequencing result

Thanks to Kyaw Myo Lwin for his contribution in this experiment. There are two mutants with no mutations in the *SMY1* locus, so I didn't assign allele number to them and left a "?" there.

myo2 sensitized strain	*smy1* allele	*smy1* nt change	*smy1* AA change
myo2-57	*smy1-2*	76 T→C 176 G→A 1217 T → C	26 C→R 59 R→H 406 I → T
	smy1-4	1062 A → G 1161 C → T 1498 T → C 1761 C → T	354 T → T (silent) 387 D → D (silent) 500 S → P 587 I → I (silent)
	smy1-5	650 T → C 713 T → C	217 V → A 238 V → A
	smy1-6	800 A → G	267 E → G
	smy1-7	839 A → G 1151 T → C	280 D → G 384 F → S
	smy1-1	190 T→C 434 T→C	64 L→L (silent) 145 L→P
myo2-47	*smy1-1*	190 T→C 434 T→C	64 L→L (silent) 145 L→P
	smy1-8	170 T→C 993 T→G	57 L→P 331 L→L (silent)
	smy1-9	508 A→C 929 C → T	170 T→P 310 A → V
	?	N/A	-
myo2-51	*smy1-10*	170 T→C	57 L→P
myo2-247	*?*	N/A	-
myo2-41	*smy1-11*	1588 A → G	529 N → D
	smy1-12	293 T→G	98 I→R
	smy1-13	515 A→G	172 D→G
	smy1-14	866 T → G	289 I → S
	smy1-3	805 T → C	269 F → L
	smy1-15	337 T→C	113 Y→H
	smy1-2	76 T→C 176 G→A 1217 T → C	26 C→R 59 R→H 406 I → T
	smy1-16	501 G→A 1087 A → G	167 M→I 363 R → R (silent)
	smy1-10	170 T→C	57 L→P
	smy1-18	875 T → G	292 L → W
	smy1-19	381 A→G 404 C→T	127 S→S (silent) 135 P→L
	smy1-20	85 G→A 352 A→G	29 E→K 118 S→G
	smy1-3	805 T → C	269 F → L

myo2-41	*smy1-21*	892 T → C	298 S → P
	smy1-22	700 A → G	234 R → G
	smy1-1	190 T→C 434 T→C	64 L→L (silent) 145 L →P

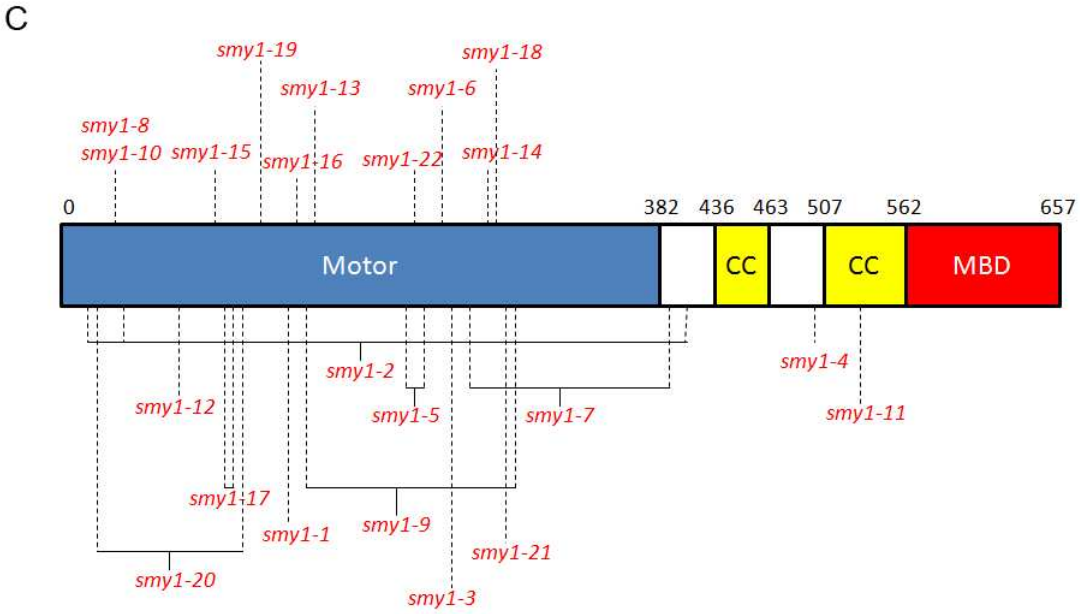


Figure 5.2 (C) Mapping mutations of *smy1* mutants to *SMY1* gene. Only non-silent mutations are shown here. *smy1-8* and *smy1-10* encode the same protein, but *smy1-8* has one silent mutation. You can see clearly the clustering of mutations in the head domain.

5.3 Characterizing the phenotype of *myo2-247 smy1-17*

Most of the *smy1* mutants were identified recently. Therefore, I have not been able to characterize their phenotype. The mutant *myo2-247 smy1-17* was identified earlier, so the results will be shown here.

First, I wanted to ask whether this *smy1-17* allele is recessive or dominant. Therefore, I transformed WT *MYO2* and *SMY1* allele into the strain. Surprisingly, none of them can fully suppress the growth defect of the *myo2-247 smy1-17* double mutant, even with overexpressed Smy1p.

Nevertheless, the temperature sensitivity is suppressed, indicating that at least this allele is not fully dominant (Fig 5.3A).

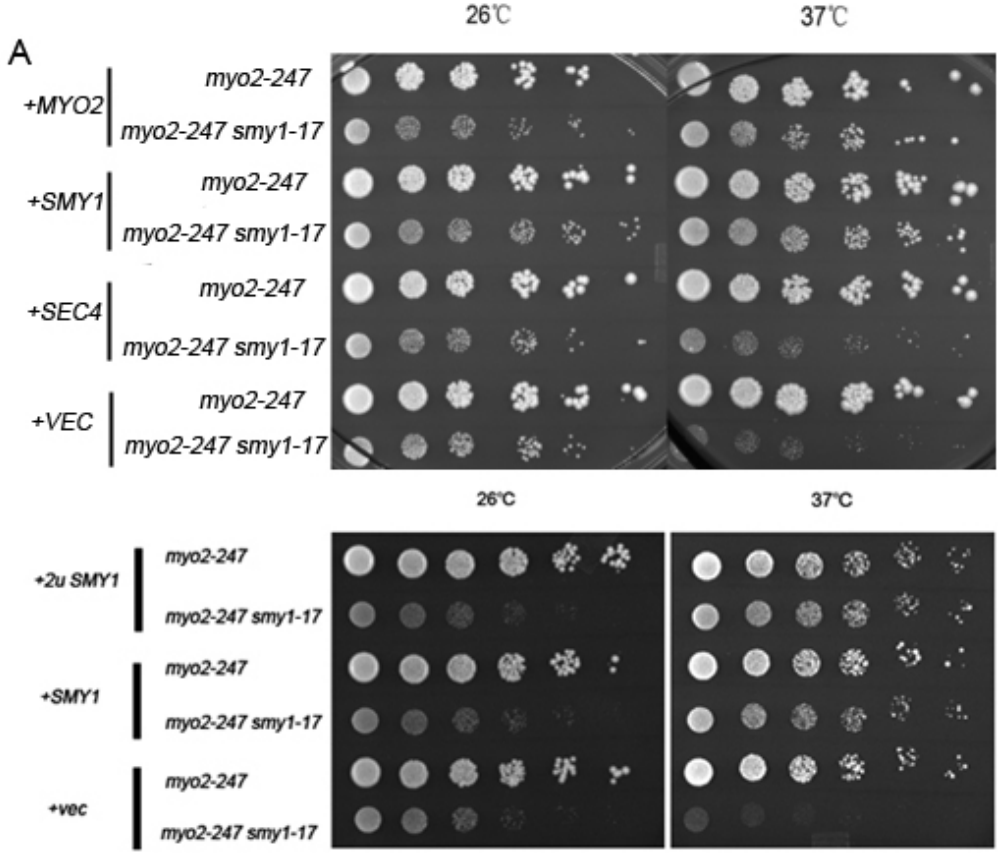


Figure 5.3 (A) suppression test of *myo2-247 smy1-17*. In either *myo2-247* or *myo2-247 smy1-17* cells, a vector carrying *MYO2, SMY1, SEC4,* multiple copy of *SMY1* or empty vector were transformed. Temperature sensitivity of this double mutant was indeed suppressed, but the strain always grew slowly.

After that, I used GFP-Sec4p as a vesicle marker to study polarized secretion. At RT, *myo2-247 smy1-17* showed a moderate phenotype compared to *myo2-247* itself. GFP-Sec4p in *myo2-247 smy1-17* cells occasional accumulated in the mother cell, while staying polarized in most cases. After a 2h shift to the restrictive temperature (37℃), I found a very dramatic change in the polarization of GFP-Sec4p in the double mutant, which became diffuse or formed bright puncta in the mother

cell. In summary, a secretion block was seen at restrictive temperature while actin cables were still intact (Fig 5.3B and C). Therefore, either Myo2p was detached from the vesicles or it had lost the ability to move its cargo while remaining binding to it. To clarify this, we need to investigate Myo2p localization. Therefore, I tried to conduct an immuno-fluorescence experiment using anti-Myo2 tail antibody in this mutant, but never succeeded. The reason was quite unexpected, that is, almost all Myo2p protein was degraded at 37℃ in *myo2-247 smy1-17* (Fig 5.3D). Since this *smy1-17* allele actually destabilizes the Myo2p protein, I feel it may not be a good mutant to work with.

Conclusion and Discussion

This chapter is the most interesting and promising part of my research, which is to study the synthetic effect of smy1 mutants combined with other mutants. Smy1p was long known to be synthetic lethal with *myo2* head and tail mutants when deleted. What's more, overexpression of Smy1p could suppress both head and tail *myo2* mutants. With the fact that they also interact with each other and travel together, Smy1p is very likely to play some important role in Myo2p's function. The hypothesis I proposed was that Smy1p actually enhances the interaction between Myo2p and secretory vesicle adaptors. In this model, it is easy to explain why overexpression of Smy1p would suppress *myo2* tail mutants, because Smy1p brings Myo2p back to secretory vesicles. However, why

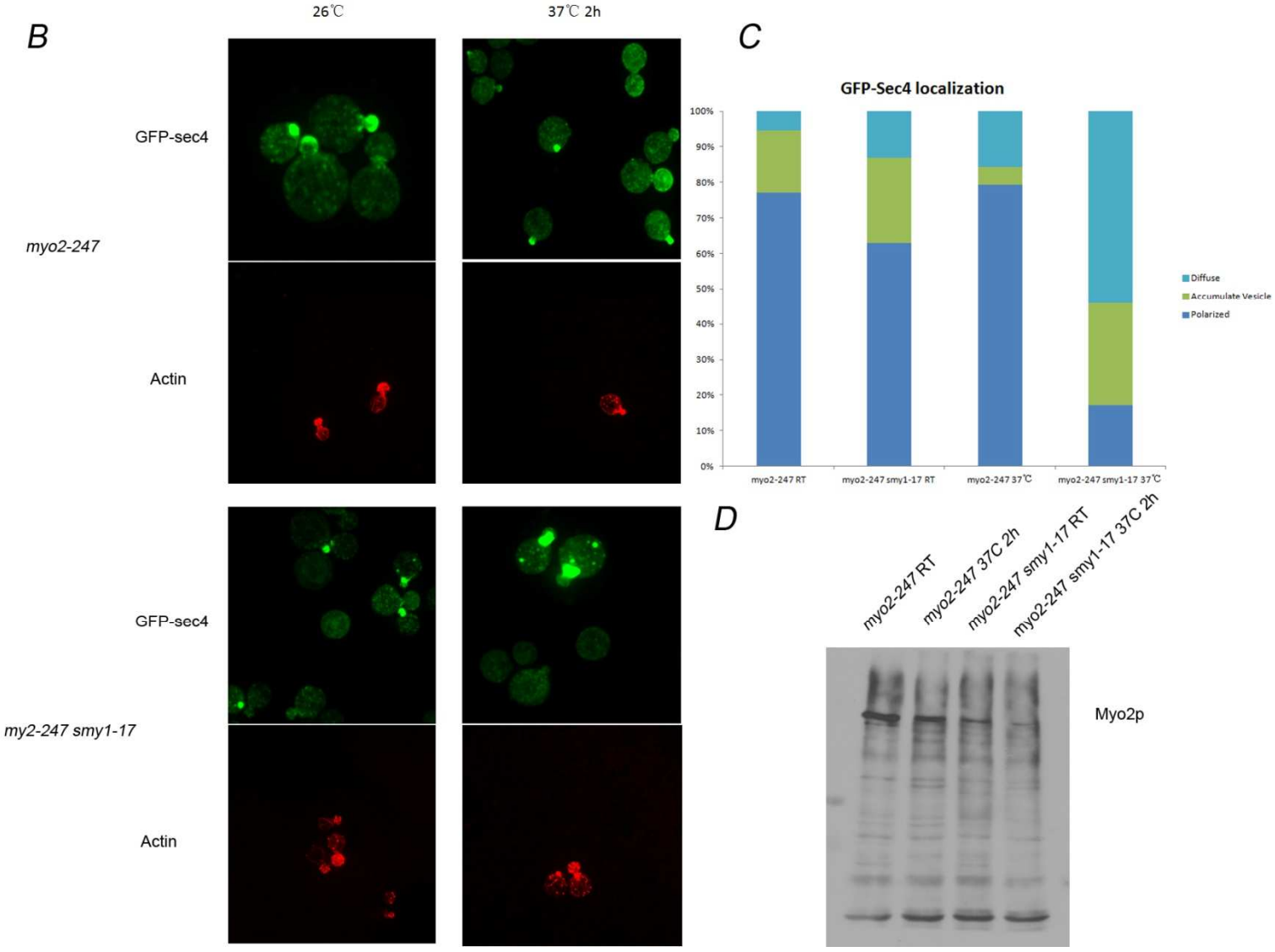


Figure 5.3 characterizing phenotype of double mutant

(B) GFP-Sec4p staining in *myo2-247* and *myo2-247 smy1-17* cells. Cells were imaged after formaldehyde fixation. *myo2-247 smy1-17* showed depolarized Sec4p staining. Actin cables were still fine, while Actin patches were located in the mother cell more frequently.

(C) Quantification of phenotype. Blue is polarized GFP-Sec4p, green represents accumulated GFP-Sec4p, and teal is diffuse. This was based on three independent experiment

(D) Myo2p is degraded in double mutant by anti-myo2 tail WB. myo2-247 was not very stable at high temperature by itself, and when became even more unstable when combined to *smy1-17.*

Smy1p could also suppress *myo2* head mutants, of which *myo2-66* was the only one known, is quite curious. One hint from previous research is that Daniel Schott once showed that *myo2-66* is easily degraded at high temperature while overexpressing Smy1p rescues it (unpublished data). If we keep that in mind and look at the result I got for *myo2-247*, which is another *myo2* head mutant, we find some similarity. *myo2-247* is also unstable at high temperature and this effect is enhanced by a mutant *smy1-17* allele. When the two mutants are combined, the cell grows very slowly and shows a severe secretion block. I didn't check other organelles, but they should also be affected. Also, it would be interesting to know where *smy1-17* localizes when we get the Smy1p antibody.

Another exciting finding from this research is that almost all the *myo2* tail mutants that are synthetic lethal with *smy1* deletion have mutations in the Rab binding sites. To support that, most of them indeed lose interaction with Sec4p. That means, when Myo2p has a problem binding to Sec4p, Smy1p becomes essential. What does that tell us? One possibility is that Smy1p actually enhances the interaction between Myo2p and Sec4p, but this could not be verified by the Y2H experiment. Maybe *in vitro* reconstitution using purified proteins would provide us with more information, as I have purified plenty of Smy1p protein. A second explanation is that, Smy1p actually bridges the interaction of Myo2p to other adaptors on secretory vesicles, of which PI4P could be a

candidate. To answer that question, raising the level of PI4P to see if it can rescue the synthetic lethality of *myo2-16 smy1Δ* would be very interesting.

The new *smy1* mutants provide a rich resource to study its function. From the sequences we got, 30 out of 34 (88%) non-silent mutations are in the head domain of Smy1p. Although we expect to see more mutations in the head domain because the homologous recombination happens in the tail region, such clustering of mutations is still surprising. One would speculate that either the head domain is very important so any mutation will have a big influence, or the tail domain is too vital for its function to have any mutation. Maybe some *in vitro* experiment like ATPase activity assay on the head domain would be helpful for us to understand this.

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

In this thesis, I mainly introduced the work I have finished in the past two years and some ongoing work right now. This chapter will be a short summary.

I started my exploration of Smy1p function by studying its localization, because I have a lot of doubt on the Trybus group's paper, which claims Smy1p to bind to secretory vesicles and acts as an electrostatic tether. Through my experiments, I showed that Myo2p binding is essential but not sufficient for Smy1p localization, which clearly disagrees with their model. What's more important, this result indicates that a secretory vesicle and Myo2p synergy exists and Smy1p probably functions as part of a large adaptor complex.

Following that, I constructed a series of Smy1p truncation constructs and tried to find a localization domain. Luckily or unluckily, I found that the localization of Smy1p is very complicated, influenced by all three domains in different ways. Together with the expression level data, I proposed that there is a balance between the head and the tail, while the coiled-coil region is a bridge or enhancer. This part is interesting but I think we are still far away from understanding the function of each domain when the function of the whole protein is unclear.

In an attempt to understand Smy1's function, I set out to create some synthetic mutants. One way to create such mutants in existing

conditional mutants is to physically get rid of Smy1p protein. I adopted a method which took advantage of the proteasome system and the controllable GAL promotor. Although the experiment didn't work in the expected way, the result was still informative. Smy1p depletion in *myo2-16* cells causes a massive secretion block, which supports our model that Smy1p functions in polarized secretion. Another ongoing project is to create *smy1 myo2* double mutants. I spent a long time refining the whole system and to manually pick the colonies. Right now we have more than 30 mutants to work with. Studying their phenotype should be enlightening and exciting.

As future directions, there are several questions I want to ask:

(1) How is Smy1p involved in polarized secretion?

All the evidence I got prompts me to propose the hypothesis that Smy1p is an accessory protein that bridges the interaction between Myo2p and a secretory vesicle. This bridging or enhancing effect is vital when Myo2p and Sec4p have problems binding to each other. However, how can we design experiment to support this idea? A Y2H experiment in which my *myo2* tail mutants and Sec4p's interaction were tested when NLS-Smy1p is expressed gave us a negative result, but the expression of NLS-Smy1p was not verified. With the Smy1p antibody available in the future, I strongly suggest to repeat that experiment. Besides, *in vitro* reconstitution has always been a promising and convincing approach.

Myo2p binding to Sec4p has been confirmed by Felipe Santiago, but its binding to PI4P and Smy1p couldn't be repeated. Further refinement of the system is needed (proportion and purity of proteins, buffer, PI4P level, etc.). In addition, physical depletion experiment in *sec* mutants would also be helpful for us to establish a model for Smy1p function.

(2) At which step does Smy1p join in vesicle transport and when does it leave?

The primary problem we need to figure out is whether Smy1p localizes to a special type of vesicle? Is Smy1p sorted or participate in sorting? To answer these questions, we have to double label Smy1p and Sec4p and study their movement carefully. Right now Smy1p-3mcherry is too dim to give a clear signal on secretory vesicles, while Smy1p-3GFP works fairly well. So a red Sec4p is definitely needed.

Besides that, we can conduct FRAP experiment to study Smy1p's life cycle. In this way, we will be able to compare its behavior to Sec4p and Myo2p and further understand its interaction. Once this is done, its life cycle in *myo2* and other mutants can be determined. Also, the mutant *smy1p* would also be interesting subjects.

(3) What exactly does Smy1p overexpression do to the cell?

Myo2p hyperpolarization was observed when Smy1p is overexpressed in WT cells. Two explanations are possible. One is that each vesicle carries more Myo2p, and the Myo2p life cycle stays the same. The other one is

that Myo2p stays in the bud for a longer time. Right now with Kirk Donovan's preliminary data, the second hypothesis seems to be favored. To further confirm this, we need to do FRAP experiment on Myo2p-3GFP when Smy1p is overexpressed or not. Comparing the two groups would solve this problem.

If Myo2p does stay longer in the bud when Smy1p is overexpressed, we want to know why that would happen. Is Sec4p locked in its GTP form for longer? Or is Myo2p binding to Sec4p enhanced so much that even GDP-bound Sec4p could still bind to Myo2p? There would be a lot of experiments to work on.

(4) What are the binding partners of Smy1p? Does Smy1p bind PI4P? Right now Myo2p is the only interacting protein that we know, but this interaction cannot be detected using *in vitro* pull-down assay, presumably because the interaction is transient or weak. Besides, Smy1p may bind to PI4P although its overexpression doesn't suppress *pik1-83*. However, this could be due to a complication caused by lower PI4P levels. Further *in vitro* reconstitution may be a good system to solve these problems. At last, what else does Smy1p bind? In vitro pull-down experiments are proposed for a long time but I didn't have the chance to try.

(5) What are the functions of each domain of Smy1p? Why is the N-terminal cleaved? Why do truncations have different expression

levels?

To investigate these problems, I believe we have to start from analyzing the mutants I identified. 88% of the mutants have mutations in the head domain, indicating the importance of it. I would suggest purifying the head domain and testing the ATPase activity. Besides, does it bind to Myo2p also? If so, an interesting model would be generated. N-terminal modification of this domain is also an intriguing question to investigate. In total, after characterizing the phenotype of the mutants, we can extend our research to the molecular level and finally solve the mystery of Smy1p.

APPENDIX: STRIN LIST

ABY#	Description	Genotype	Source
1655	WT	MATa *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	Felipe Santiago-Tirado
2702	*myo2-12*	MATa *myo2-12::HIS3 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	Felipe Santiago-Tirado
2704	*myo2-14*	MATa *myo2-14::HIS3 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	Felipe Santiago-Tirado
2705	*myo2-16*	MATa *myo2-16::HIS3 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	Felipe Santiago-Tirado
?	*myo2-24*	MATa *myo2-24::HIS3 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	Irina Chernyakov
511	*sec2-56*	MATx *sec2-56 ura3-52 leu2-3,112*	Felipe Santiago-Tirado
?	*sec4-8*	MAT? *sec4-8 ura3-52 leu2-3,112*	Felipe Santiago-Tirado
?	Y8830	?	Haiyuan Yu
?	Y8910	?	Haiyuan Yu
3304	WT Smy1-3HA	MATa Smy1-3HA::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3305	*myo2-14* Smy1-3HA	MATa *myo2-14::HIS3* Smy1-3HA::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3306	*myo2-16* Smy1-3HA	MATa *myo2-16::HIS3* Smy1-3HA::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3307	*myo2-247 trp1Δ*	MATa *myo2-247::HIS3 trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3308	*myo2-247 smy1-17*	MATa *myo2-247::HIS3 trp1Δ*::KanR *smy1-17:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3309	*myo2-16 bni1Δ*	MATa *myo2-16::HIS3 bni1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3310	*myo2-16 bnr1Δ*	MATa *myo2-16::HIS3 bnr1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3311	*trp1Δ*	MATx *trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0*	this study
3312	*smy1-17*	MATx *trp1Δ*::KanR *smy1-17:: TRP1 his3Δ1*	this study

		ura3Δ0 leu2Δ0 lys2Δ0	
3313	*trp1Δ*	MATa *trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3314	*myo2-16 smy1Δ* 415 UBI-R-Smy1	MATa *myo2-16::HIS3 smy1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0* pRS415 UBI-R-Smy1-6his	this study
3315	*trp1Δ myo2-47*	MATa *myo2-47::HIS3 trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3316	*trp1Δ myo2-57*	MATa *myo2-57::HIS3 trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3317	*trp1Δ myo2-51*	MATa *myo2-51::HIS3 trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3318	*trp1Δ myo2-43*	MATa *myo2-43::HIS3 trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3319	*trp1Δ myo2-41*	MATa *myo2-41::HIS3 trp1Δ*::KanR *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3320	*myo2-41 smy1-15*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-15:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3321	*myo2-41 smy1-2*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-2:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3322	*myo2-41 smy1-16*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-16:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3323	*myo2-47 smy1-1*	MATa *myo2-47::HIS3 trp1Δ*::KanR *smy1-1:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3324	*myo2-47 smy1-8*	MATa *myo2-47::HIS3 trp1Δ*::KanR *smy1-8:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3325	*myo2-47 smy1-9*	MATa *myo2-47::HIS3 trp1Δ*::KanR *smy1-9:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3326	*myo2-47 smy1-?*	MATa *myo2-47::HIS3 trp1Δ*::KanR *smy1-?:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3327	*myo2-57 smy1-2*	MATa *myo2-57::HIS3 trp1Δ*::KanR *smy1-2:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3328	*myo2-57 smy1-4*	MATa *myo2-57::HIS3 trp1Δ*::KanR *smy1-4:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3329	*myo2-57*	MATa *myo2-57::HIS3 trp1Δ*::KanR	this study

	smy1-5	*smy1-5:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	
3330	*myo2-57 smy1-6*	MATa *myo2-57::HIS3 trp1Δ*::KanR *smy1-6:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3331	*myo2-41 smy1-3*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-3:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3332	*myo2-41 smy1-21*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-21:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3333	*myo2-41 smy1-22*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-22:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3334	*myo2-41 smy1-1*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-1:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3335	*myo2-41 smy1-11*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-11:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3336	*myo2-41 smy1-12*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-12:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3337	*myo2-41 smy1-13*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-13:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3338	*myo2-41 smy1-14*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-14:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3339	*myo2-57 smy1-7*	MATa *myo2-57::HIS3 trp1Δ*::KanR *smy1-7:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3340	*myo2-57 smy1-1*	MATa *myo2-57::HIS3 trp1Δ*::KanR *smy1-1:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3341	*myo2-57 smy1-10*	MATa *myo2-57::HIS3 trp1Δ*::KanR *smy1-10:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3342	*myo2-41 smy1-20*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-20:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3343	*myo2-41 smy1-10*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-10:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study

3344	*myo2-41* *smy1-18*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-18:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3345	*myo2-41* *smy1-19*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-19:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3346	*myo2-247* *smy1-?*	MATa *myo2-247::HIS3 trp1Δ*::KanR *smy1-?:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study
3347	*myo2-41* *smy1-3*	MATa *myo2-41::HIS3 trp1Δ*::KanR *smy1-3:: TRP1 his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	this study

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